

**TTP-RELATED ZINC FINGER DOMAINS AND METHODS OF USE**

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This application claims benefit of priority from U.S. Serial No. 60/148,810, filed August 13, 1999, herein incorporated by reference in its entirety.

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**ACKNOWLEDGMENTS**

This invention was made with intramural support from the National Institutes of Health. The government has certain rights in the invention.

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**FIELD OF THE INVENTION**

This invention relates generally to regulation of mRNA stability, for example, to stimulate or inhibit the production of polypeptides involved in disease.

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**BACKGROUND OF THE INVENTION**

The zinc finger protein family encompasses a broad variety of proteins with assorted functions. One relatively uncommon class of zinc finger proteins contains 20 fingers of the Cys-Cys-Cys-His (CCCH) type, in which three cysteines and one histidine are thought to coordinate a single atom of zinc. Members of a very small subclass of the larger family of CCCH zinc finger proteins contain two tandem zinc fingers consisting of  $Cx_8Cx_5Cx_3H$  (wherein "x" refers to variable amino acids), spaced exactly 18 amino acids apart. The prototype of proteins of this CCCH double zinc 25 finger subclass is tristetraprolin (TTP), also known as TIS11 and Nup475. TTP is localized to the nucleus of quiescent fibroblasts, but is rapidly phosphorylated on serine residues and translocated to the cytosol after stimulation with serum or other mitogens. TTP is almost completely cytosolic in macrophages.

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TTP-deficient mice appear normal at birth, but rapidly develop a wasting syndrome accompanied by erosive arthritis, dermatitis, alopecia, autoantibodies and myeloid hyperplasia. Essentially all of these inflammation-associated conditions can be prevented by the injection of monoclonal antibodies specific for mouse tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), one of the principal mediators of the inflammatory response in mammals.

In addition to its well known role in acute septic shock, TNF- $\alpha$  has been implicated in the pathogenesis of chronic processes such as autoimmunity, graft-versus-host disease, rheumatoid arthritis, Crohn's disease, and the cachexia that accompanies cancer and acquired immunodeficiency syndrome (AIDS). Anti-TNF- $\alpha$  therapies such as neutralizing antibodies against TNF- $\alpha$  and chimeric soluble TNF- $\alpha$  receptors have demonstrated efficacy against some of these conditions in clinical trials.

The present invention is based upon the discovery that the TTP tandem zinc finger domain alone (as well as the analogous domain from other TTP-related polypeptides) is sufficient to bind to an AU-rich element (ARE) within the 3' untranslated region (UTR) of a TNF- $\alpha$  mRNA molecule, thereby targeting the TNF- $\alpha$  mRNA molecule for destruction. The present invention is further based upon the discovery that this mRNA turnover mechanism is not unique to TNF- $\alpha$  mRNA, as destruction of other mRNAs containing AREs, such as the mRNA that encodes granulocyte-macrophage stimulating factor (GM-CSF), is stimulated by the tandem zinc finger domain of TTP and TTP-related polypeptides. Accordingly, the invention provides functional fragments of TTP and TTP-related polypeptides, nucleic acids encoding such functional fragments, and methods of using the nucleic acids and polypeptide fragments to regulate (i.e., stimulate or inhibit) the destruction of mRNAs that contain an ARE. These methods may be used to treat diseases and conditions that are affected by polypeptides encoded by an mRNA molecule whose destruction is modulated by an ARE. The polypeptides and methods of the invention can also be used to identify compounds that regulate the activity of TTP and TTP-related

polypeptides, and are therefore useful for regulating levels of mRNA encoding TNF- $\alpha$ , GM-CSF, and other mRNAs containing functional AREs, such as that encoding interleukin-3 (IL-3).

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## SUMMARY OF THE INVENTION

Described herein are methods based upon the discovery that TTP and TTP-related proteins stimulate the destruction of certain mRNAs by binding to an AU-rich element (ARE) within the 3' untranslated region of such mRNAs, and that the zinc  
10 finger domain of TTP and TTP-related proteins is sufficient to mediate this destruction.

In a first aspect, the invention features a method of treating granulocytopenia in a subject, including administering to the subject an agent that inhibits the degradation of GM-CSF mRNA, thereby treating granulocytopenia in the subject.

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In various embodiments of the first aspect of the invention, the granulocytopenia is relative or absolute; the degradation of GM-CSF mRNA is inhibited by inhibiting the mRNA degradative activity of TTP; or the agent that inhibits the degradative activity of TTP is a competitor of TTP. For example, the competitor  
20 can compete with TTP for binding on the AU-rich element (ARE) of GM-CSF mRNA; or the competitor can compete with TTP for binding on an mRNA degradative enzyme.

In other embodiments of the first aspect of the invention, the degradation of GM-CSF mRNA is inhibited by inhibiting the mRNA degradative activity of ERF1; for  
25 example, the agent that inhibits the mRNA degradative activity of ERF1 can be a competitor of ERF1. The competitor can compete with ERF1 for binding on the AU-rich element (ARE) of GM-CSF mRNA; or can compete with ERF1 for binding on an mRNA degradative enzyme.

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In still other embodiments of the first aspect of the invention, the degradation of GM-CSF mRNA is inhibited by inhibiting the mRNA degradative activity of ERF2. The agent that inhibits the mRNA degradative activity of ERF2 can be a competitor of ERF2. For example, the competitor can compete with ERF2 for binding on the AU-rich element (ARE) of GM-CSF mRNA, or can compete with ERF2 for binding on an mRNA degradative enzyme.

In a second aspect, the invention features a method of treating granulocytopenia in a subject, including administering to the subject a mutant TTP that has reduced activity compared to wild type TTP. In various embodiments of the second aspect of the invention, the activity of TTP reduced is degradation of GM-CSF mRNA; or the mutant TTP is administered by delivering to the subject a nucleic acid that encodes the mutant TTP and allows expression of the mutant TTP in cells of the subject.

In a third aspect, the invention features a method of treating granulocytopenia in a subject, including administering to the subject a mutant ERF1 that has reduced activity compared to wild type TTP. In various embodiments of the third aspect of the invention, the activity of ERF1 reduced is degradation of GM-CSF mRNA; or the mutant ERF1 is administered by delivering to the subject a nucleic acid that encodes the mutant ERF1 and allows expression of the mutant ERF1 in cells of the subject.

In a fourth aspect, the invention features a method of treating granulocytopenia in a subject, including administering to the subject a mutant ERF2 that has reduced activity compared to wild type TTP. In various embodiments of the fourth aspect of the invention, the activity of ERF2 reduced is degradation of GM-CSF mRNA, or the mutant ERF2 is administered by delivering to the subject a nucleic acid that encodes the mutant ERF2 and allows expression of the mutant ERF2 in cells of the subject.

In a fifth aspect, the invention features a mutant TTP that has a reduced TTP activity compared to wild type TTP. In various embodiments of the fifth aspect of the

invention, the activity of TTP reduced is TTP binding to the ARE of GM-CSF mRNA; the activity of TTP reduced is TTP binding to an mRNA degradative enzyme; the activity of TTP reduced is degradation of GM-CSF mRNA; the mutant is C124R; or the mutant is C147R.

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In a sixth aspect, the invention features a mutant ERF1 that has a reduced ERF1 activity compared to wild type ERF1. In various embodiments of the sixth aspect of the invention, the activity of ERF1 reduced is ERF1 binding to the ARE of GM-CSF mRNA; the activity of ERF1 reduced is ERF1 binding to an mRNA degradative  
10 enzyme; or the activity of ERF1 reduced is degradation of GM-CSF mRNA.

In a seventh aspect, the invention features a mutant ERF2 that has a reduced ERF2 activity compared to wild type ERF2. In various embodiments of the seventh aspect of the invention, the activity of ERF2 reduced is ERF2 binding to the ARE of  
15 GM-CSF mRNA; the activity of ERF2 reduced is ERF2 binding to an mRNA degradative enzyme; or the activity of ERF2 reduced is degradation of GM-CSF mRNA.

In an eighth aspect, the invention features a method of screening an agent for  
20 the ability to inhibit an activity of TTP, including the steps of: a) cotransfecting a cell with a nucleic acid that encodes TTP and a nucleic acid that includes an ARE downstream of a nucleic acid sequence encoding a reporter protein; b) contacting the cell of step a) with the agent; and c) comparing the expression of the reporter protein in the cell of step b) to the cell of step a) in the absence of the agent, an increase in  
25 reporter gene expression in the cells of step b) compared to the cells of step a) indicating that the agent has the ability to inhibit an activity of TTP.

In a ninth aspect, the invention features a method of screening an agent for the ability to compete with TTP for binding to the ARE of mRNA, including the steps of:  
30 a) transfecting a cell with a nucleic acid that encodes TTP; b) obtaining a cytosolic

extract of the cell of step a); c) contacting the cytosolic extract of step b) with the agent; d) contacting the cytosolic extract of steps b) and c) with a probe including an ARE; e) comparing the binding of the probe to TTP in the cytosolic extract of step b) with the binding of the probe to TTP in the cytosolic extract of step c), the presence of  
5 reduced binding of the probe to TTP in the cytosolic extract of step c) indicating an agent that can compete with TTP for binding to the ARE of mRNA.

In a tenth aspect, the invention features a method of stimulating the degradation of an mRNA molecule having an AU-rich element (ARE), including contacting the  
10 mRNA molecule with a tandem zinc finger (TZF) polypeptide consisting essentially of the tristetraprolin (TTP) zinc finger domain or including a TTP-like zinc finger domain, thereby stimulating degradation of the mRNA molecule.

In various embodiments of the tenth aspect of the invention, the TTP-like zinc  
15 finger domain is selected from the ERF1 zinc finger domain, the ERF2 zinc finger domain, and the XC3H-4 zinc finger domain; the TZF polypeptide is selected from ERF1, ERF2, and XC3H-4; the mRNA molecule is within a cytosolic extract; the mRNA molecule is within a cell; the mRNA molecule is within a patient or subject; the mRNA molecule encodes TNF- $\alpha$ ; or production of a polypeptide (e.g., TNF- $\alpha$ )  
20 encoded by the mRNA molecule is decreased.

In other embodiments of the tenth aspect of the invention, the TZF polypeptide is administered to a patient or subject to treat, inhibit, or prevent a TNF- $\alpha$ -related disease or condition in the patient or subject; a nucleic acid encoding the TZF  
25 polypeptide is administered to a patient or subject to treat, inhibit, or prevent a TNF- $\alpha$ -related disease or condition in the patient or subject; or the ARE is a class II ARE.

In an eleventh aspect, the invention features a method of identifying a compound that modulates the activity of TTP or a TTP-like polypeptide, including: a)  
30 contacting a sample with the compound, and b) detecting or measuring the binding

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between an ARE and a TZF polypeptide consisting essentially of a TTP zinc finger domain or a polypeptide including a TTP-like zinc finger domain in the sample, whereby an increase or decrease in the binding between the ARE and the polypeptide, relative to the binding between the ARE and the polypeptide in the sample not  
5 contacted with the compound, identifies a compound that modulates the activity of TTP or a TTP-like polypeptide.

In various embodiments of the eleventh aspect of the invention, an increase in the binding between the ARE and the polypeptide identifies a compound that stimulates  
10 the activity of a TTP or a TTP-like polypeptide; the method identifies a compound that stimulates degradation of an mRNA molecule including an ARE; the mRNA molecule encodes TNF- $\alpha$ ; a decrease in the binding between the ARE and the TZF polypeptide identifies a compound that inhibits the activity of TTP or a TTP-like polypeptide; the method identifies a compound that inhibits degradation of an mRNA molecule  
15 including an ARE; the mRNA molecule encodes GM-CSF or IL-3; the method further includes contacting the sample with an inhibitor of mRNA transcription prior to detecting or measuring the binding between the ARE and the TZF polypeptide; or the ARE is a class II ARE.

20 In a twelfth aspect, the invention features a method of identifying a compound that mimics the activity of TTP or a TTP-like polypeptide, including: a) contacting a first sample including an RNA molecule including an ARE with a compound; b) contacting a second sample including an RNA molecule including an ARE with the compound and with a saturating amount of a TZF polypeptide consisting essentially of  
25 a TTP zinc finger domain or including a TTP-like zinc finger domain; c) detecting or measuring degradation of the RNA molecule or binding of the compound to the ARE in the first sample and in the second sample; e) comparing the degradation or binding in the first sample to the degradation or binding in the first sample not contacted with the compound; and f) comparing the degradation or binding in the first sample to the  
30 degradation or binding in the second sample, whereby an increase in degradation or

binding in the first sample contacted with the compound relative to the sample not contacted with the compound, and lack of an increase in degradation or binding in the second sample contacted with the compound and with the saturating amount of a TZF polypeptide, identifies a compound that mimics the activity of TTP or a TTP-like peptide. In one embodiment of the eleventh aspect of the invention, the ARE is a class II ARE.

In a thirteenth aspect, the invention features a polypeptide consisting essentially of a TTP zinc finger domain or a TTP-like zinc finger domain. In various embodiments of the twelfth aspect of the invention, the polypeptide can bind to a class II ARE within an mRNA molecule and stimulate degradation of the mRNA molecule under physiological conditions, or the TTP-like zinc finger domain is from ERF1, ERF2, or XC3H-4.

In a fourteenth aspect, the invention features a nucleic acid consisting essentially of a nucleotide sequence that encodes a TTP zinc finger domain or a TTP-like zinc finger domain. In various embodiments of the thirteenth aspect of the invention, the TTP-like zinc finger domain is from ERF1, ERF2, or XC3H-4.

In a fifteenth aspect, the invention features a vector including the nucleic acid of claim 66, wherein the nucleic acid is operably linked to a promoter for transcription of the nucleic acid.

In a sixteenth aspect, the invention features a mutant XC3H-4 that has a reduced XC3H-4 activity compared to wild type XC3H-4. In various embodiments of the seventh aspect of the invention, the activity of XC3H-4 reduced is XC3H-4 binding to the ARE of GM-CSF mRNA; the activity of XC3H-4 reduced is XC3H-4 binding to an mRNA degradative enzyme; or the activity of XC3H-4 reduced is degradation of GM-CSF mRNA.



In this specification and in the claims that follow, reference is made to a number of terms that shall be defined to have the following meanings.

As used in the specification and the appended claims, the singular forms "a,"  
5 "an," and "the" include plural referents unless the context clearly dictates otherwise.

In the present invention "comprising" means that at least the elements specified are present. By "containing" is meant that at least the elements specified are present.

10 By "TTP zinc finger" or "TTP zinc finger domain" is meant a polypeptide fragment of 77 amino acids or less, which has a 64 amino acid sequence identical to the sequence in TTP that contains two CCCH zinc fingers spaced eighteen amino acids apart, as shown in Fig. 5B, and which, by itself, is sufficient to bind to a class II ARE within an mRNA molecule and stimulate degradation of the mRNA molecule.

15 By "TTP-like zinc finger" or "TTP-like zinc finger domain" is meant a polypeptide fragment that has a 64 amino acid TZF consensus sequence as set forth below, or as shown in Fig. 5B, which is not identical to a TTP zinc finger, and which, by itself, is sufficient to bind to a class II ARE within an mRNA molecule and  
20 stimulate degradation of the mRNA molecule.

By "class II ARE" is meant an AU-rich region in the 3'-untranslated region of an mRNA molecule, which contains repeats of the pentanucleotide "AUUUA" sufficient to permit binding of TTP or a TZF polypeptide to stimulate deadenylation  
25 and/or degradation of the mRNA molecule. An example of a class II ARE is depicted, e.g., in Fig. 1. Class II AREs are known in the art, and are described, e.g., in Xu et al. *Mol. Cell. Biol.*, 17:4611-4621, 1997.

By "TTP-like polypeptide" or "TZF polypeptide" is meant a polypeptide that  
30 displays TTP-like activity, i.e., can bind to a class II ARE and can stimulate

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deadenylation and/or degradation of an mRNA molecule containing a class II ARE. A TTP-like polypeptide can be a polypeptide consisting of only the 64 amino acid tandem zinc finger (TZF) domain having the TZF amino acid consensus sequence (representatives of which are shown in Fig. 5B), or a TZF polypeptide can be a larger  
 5 polypeptide comprising the TZF domain (for example, a naturally occurring polypeptide such as ERF1 or ERF2), or the TZF polypeptide may contain the TZF domain plus additional amino acid sequences, as long as the TTP-like polypeptide can carry out its TTP-like activities of binding a class II ARE and stimulating mRNA deadenylation and/or degradation. A polypeptide having the amino acid sequence of  
 10 full-length human TTP or full-length rodent TTP is excluded from this definition.

A TTP-like polypeptide or TZF polypeptide will contain a 64 amino acid TZF domain having a TZF consensus sequence as follows (see Fig. 5B for numbering strategy; slashes indicate alternative residues; preferred amino acids are denoted by  
 15 upper case letters and less preferred amino acids are denoted by lower case letters):

aa1-aa7:	RYKTELC
aa8:	R/s
aa9:	P/T/r
20 aa10:	F/Y
aa11:	E/S/a
aa12:	E
aa13:	S/N/t/s
25 aa14:	G
aa15:	A/R/T/S/f
aa16:	C
aa17:	K/R/a
aa18:	Y
30 aa19:	G/a/r

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	aa20:	E/A/D/n
	aa21:	K/R
	aa22-aa27:	CQFAHG
5	aa28:	L/F/I/p/k
	aa29:	H/G/i/s
	aa30:	E/D
	aa31:	L
10	aa32:	R/H
	aa33:	S/Q/v/e/p
	aa34:	L/A/P
	aa35:	T/N/s/v
15	aa36:	R/q
	aa37-aa45:	HPKYKTELC
	aa46:	R/H
	aa47:	T/K/s
20	aa48:	F/Y
	aa49:	H/Y
	aa50:	T/L/n/v
	aa51:	I/Q/a/l/y
	aa52:	G
25	aa53:	F/R/y/e/t
	aa54:	C
	aa55:	P/v/n
	aa56:	Y
30	aa57:	G/v

aa58: P/S/t/l  
aa59-aa60: RC  
aa61: H/l/n  
aa62: F  
5 aa63: I/v  
aa64: H

Examples of naturally occurring TTP-like (TZF) polypeptides include, but are not limited to: ERF1/CMG1; ERF2/TIS11D/XC3H-3.2; XC3H-3.1; XC3H-1; CTH1  
10 (carp); CTH1 (zebrafish); and XC3-H-4.

By "sample" is meant an animal (e.g., a human or non-human primate, or a domestic, farm, or laboratory animal, such as a horse, dog, cat, bird, ferret, cow, pig, sheep, goat, rat, mouse, rabbit, guinea pig, fish, or frog); a tissue, organ, or body fluid  
15 obtained from an animal; a cell (either within an animal or taken directly from an animal, or a cell maintained in culture or from a cultured cell line); a lysate, lysate fraction, or extract (e.g., a cytosolic extract) derived from a cell; a molecule derived from a cell or cellular material (e.g., a polypeptide or nucleic acid molecule); or an experimental reaction mixture (e.g., containing a buffer and salts, substrates, and/or any  
20 other molecules needed to carry out an assay) which is to be assayed or analyzed according to the methods of the invention, for example, to identify a compound that modulates the activity of TTP or a TTP-like polypeptide.

By "modulates the activity of TTP or a TTP-like polypeptide" is meant a  
25 compound that increases or decreases the binding of TTP or a TTP-like polypeptide (or a TZF polypeptide) to an ARE, e.g. (but not limited to), by binding to either the polypeptide, to an ARE, or to an RNA molecule containing the ARE, or by increasing or decreasing the amount of TTP or TTP-like polypeptide available for binding (e.g., by increasing or decreasing degradation of the polypeptide). The compound either  
30 facilitates or blocks binding between the polypeptide and the ARE, thereby stimulating

or inhibiting degradation of an mRNA molecule that contains an ARE, and that undergoes degradation that is enhanced by the binding of TTP or a TTP-like polypeptide to the ARE.

5 By "binding between an ARE and a TZF polypeptide" is meant that the ARE and TZF polypeptide physically interact with each other. The amount of binding can be detected and/or measured by methods described herein or known in the art, either directly (e.g., by gel shift or UV cross-linking assays) or indirectly (e.g., by measuring RNA levels by Northern or RNA dot blot analysis to ascertain the relative amount of  
10 mRNA degradation resulting from binding between an ARE within the 3' UTR of an RNA molecule and the TZF polypeptide; or by measuring protein levels by Western analysis or ELISA or reporter enzyme assay to ascertain the relative amount of protein, thus inferring the relative amount of mRNA degradation resulting from binding between an ARE within the 3' UTR of an RNA molecule and the TZF polypeptide).

15 By "TNF- $\alpha$ -related disease or condition" is meant any disease or condition in which TNF- $\alpha$  plays a role, and in which a decrease in TNF- $\alpha$  would be useful in treating preventing, or slowing the disease. Examples of TNF- $\alpha$ -related diseases or conditions include, but are not limited to: acute septic shock, autoimmunity, graft-  
20 versus-host disease, rheumatoid arthritis, Crohn's disease, cachexia associated with cancer or AIDS, wasting syndrome, dermatitis, alopecia, myeloid hyperplasia, and, in general, TNF- $\alpha$ -dependent inflammation.

By "a saturating amount of a TZF polypeptide" is meant an amount of a TZF  
25 polypeptide sufficient to induce the maximal amount of degradation of an RNA species that has a class II ARE and whose degradation is stimulated by a TZF polypeptide. The saturating amount of a TZF polypeptide may be in a cell or in a cell-free mixture, e.g., a cytosolic extract or other reaction mixture for identifying compounds that mimic, stimulate, or inhibit the activity of TTP or a TTP-like polypeptide.

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## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A is a diagram of a UV cross-linking assay showing cross-linking of human TTP with mouse TNF $\alpha$  mRNA ARE probes.

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Fig. 1B is a diagram showing the nucleotide sequence of the mouse TNF $\alpha$  ARE probes used in the experiment depicted in Fig. 1A.

Fig. 2 is a diagram showing a sequence alignment comparing human TTP to two human TTP-like proteins, ERF1 and ERF2.

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Fig. 3A is a diagram of a UV cross-linking assay showing cross-linking of TTP and TTP-related proteins with mouse TNF $\alpha$  mRNA ARE probes containing point mutations.

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Fig. 3B is a is a diagram showing the nucleotide sequence of the mouse TNF $\alpha$  ARE probes used in the experiment depicted in Fig. 3A.

Fig. 4 is a diagram showing a sequence alignment comparing the TTP-like frog protein XC3H-4 to two related proteins from fish, i.e., carp CTH1 and zebrafish CTH1.

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Fig. 5A is a diagram of a dendrogram showing the relatedness of amino acid sequences from the 64 amino acid tandem zinc finger domains of TTP and TTP-like polypeptides from various species.

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Fig. 5B is a diagram of a sequence alignment of the 64 amino acid tandem zinc finger domain of TTP and TTP-related polypeptides.

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## DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of the invention and the Examples included therein.

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Before the present compounds and methods are disclosed and described, it is to be understood that this invention is not limited to specific proteins or specific methods. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

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Described herein are methods based upon the discovery that tristetraprolin (TTP) binds to and destabilizes mRNAs containing a class II AU-rich element (ARE) in their 3' untranslated regions (UTRs). The mRNAs encoding polypeptides such as tumor necrosis factor-alpha (TNF- $\alpha$ ), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-3 (IL-3) all contain such AREs and are destabilized by the binding of TTP to the ARE. Described herein are methods for beneficially stimulating or inhibiting degradation of mRNAs containing AREs and methods for identifying additional compounds that are useful for regulating degradation of ARE-containing mRNAs that encode polypeptides involved in disease and inflammation.

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Experiments described herein show that TTP binds directly to the ARE of TNF- $\alpha$  mRNA. The integrity of both zinc fingers is required for this direct protein-RNA interaction, since a single mutation of cysteine to arginine within the CCCH motif from either finger abolished TTP's ARE binding activity. The same mutations abrogated the ability of TTP to destabilize the TNF mRNA in intact cells. These experiments show that TTP binds directly to the TNF ARE and destabilizes TNF mRNA in a zinc-finger dependent manner, apparently by initially stimulating mRNA deadenylation.

Two other polypeptides bearing structural similarity to TTP of this subclass have been identified to date in mammals: cMG1 (TIS11b, ERF1, Berg-36) (Barnard et

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al., *Nucleic Acids Res* 21:3580, 1993; Gomperts et al., *Oncogene* 5:1081-1083, 1990; Ning et al., *Biochem Soc Trans* 25:306S, 1997; and Varnum et al., *Mol Cell Biol* 11:1754-1758, 1991); and TIS11d (ERF2) (Varnum et al, *supra*; and Nie et al., *Gene* 152:285-286, 1995). Like TTP, ERF1/TIS11b and ERF2/TIS11d contain the two  
5 typical CCCH fingers, spaced 18 amino acids apart, with the sequence RYKTEL or a variant leading into each finger. Proteins with nearly identical double zinc fingers spaced 18 amino acids apart also have been identified in *Drosophila* and yeast (Ma et al., *Oncogene* 9:3329-3334, 1994; Ma and Herschman, *Oncogene* 10:487-494, 1995; and Thompson et al., *Gene* 174:225-233, 1996).

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In addition to the *Xenopus* homologues (see Fig. 5B) of the three mammalian proteins described above, which all contain two tandem zinc fingers (TZFs), also known is a fourth *Xenopus* homologue (XC3H-4) containing two CCCH zinc fingers spaced 18 amino acids apart and preceded by the R(K)YKTEL sequence, as well as an  
15 additional more carboxyl-terminal pair of CCCH zinc fingers that are more closely spaced and lack the lead-in R(K)YKTEL sequence (De et al., *Gene* 228:133-145, 1999).

The mammalian and *Xenopus*/fish CCCH proteins described herein comprise  
20 the known members of a subclass of vertebrate CCCH proteins in which the following features characterize the tandem zinc finger (TZF) domains: 1) Both fingers within the TZF domain in the four proteins are preceded by a conserved six amino acid lead-in sequence, R(K)YKTEL; 2) Both fingers in all proteins contain the following conserved residues and spacing, Cxx(F/Y)x3GxCxYxx(K/R)CxFxH, where x represents variable  
25 amino acids; 3) Both fingers in all proteins are separated by exactly 18 amino acids, i.e., between the terminal H of the first finger and the first C of the second finger. These characteristics are identical in the protein homologues despite their species of origin, ranging from human to *Xenopus laevis*; 4) The three mammalian proteins and their homologues are basic, with overall pIs ranging from 8.75-9.91. In contrast, the  
30 *Xenopus* protein XC3H-4 has an overall pI of 5.9; however, the TZF domain itself is



basic, with a pI of 9.1, similar to the pIs of the TZF domains from the other three proteins (De et al., *Gene* 228:133-145, 1999).

As shown herein, the two other known mammalian members of this class of  
5 tandem CCCH zinc finger proteins exhibited activities similar to TTP in various assays. Specifically, the rat cMG1 protein, whose homologues include mouse TIS11b, human ERF1, and *Xenopus* XC3H-2, and the *Xenopus* XC3H-3 protein, whose homologues include mouse TIS11d and human ERF2, stimulated the apparent deadenylation and destruction of the TNF mRNA in intact cell transfection experiments. Second, these  
10 proteins, as well as a TZF domain from the fourth known vertebrate member of this protein class, *Xenopus* XC3H-4, bound to the ARE of the TNF mRNA in cell-free experiments, as demonstrated by gel shift and UV-light cross-linking experiments. Finally, a 77 amino acid TTP fragment containing the 64 amino acid TZF domain of TTP was sufficient to mediate the TTP effect on TNF mRNA stability in cell  
15 transfection experiments, as well as in cell-free gel shift and crosslinking experiments.

In contrast, the hU2AF35 protein exhibited no activity in these assays. This protein, a subunit of the essential mRNA splicing factor hU2AF, contains two related CCCH zinc fingers, present in the protein homologues from human to yeast, but that  
20 are separated by 116 amino acids in the human protein. Thus, the mere presence of two zinc fingers of this type does not appear to confer TNF mRNA destabilizing and ARE binding activity, in the absence of the other characteristics of the more closely spaced TZFs of TTP and its relatives.

25 The finding that TTP and its related proteins all exhibited similar mRNA binding and destabilizing activities indicates that the domain they all hold in common, the 64 amino acid TZF domain, is critical for these activities. That the TZF domain is necessary for these activities is demonstrated by the finding, described herein, that mutation of a single cysteine in either zinc finger to an arginine completely abrogated  
30 TTP's ARE-binding and mRNA destabilizing activities. Therefore, although not

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wishing to be bound by theory, it appears likely that the first step in the effect of TTP to destabilize these mRNAs is its binding to the ARE, since non-binding zinc finger mutants do not stimulate TNF mRNA breakdown in intact cells.

5           The present experiments demonstrate that a 77 amino acid domain of TTP (SEQ ID NO: 23) that contains the 64 amino acid TZF domain was also sufficient for both ARE binding and TNF mRNA destabilizing activities. Therefore, at present, this 64 amino acid domain is the minimum known TTP sequence required for these activities. The corresponding minimum known ARE sequence required for this binding is 24  
10       bases (nucleotides 1309-1332 in Genbank accession number X02611). Therefore, polypeptides containing TZF domains as described herein may be used in the methods of the invention to treat diseases and conditions involving in which it would be beneficial to regulate destruction of an mRNA molecule whose degradation is stimulated by an ARE. The polypeptides and methods of the invention can also be  
15       used to identify compounds that regulate the activity of TTP and TTP-related polypeptides, and are therefore useful for regulating levels of mRNA encoding TNF- $\alpha$ , GM-CSF, and other mRNAs containing functional AREs, such as that encoding interleukin-3 (IL-3). Useful assays for identifying such compounds are described herein.

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As demonstrated herein, TTP deficiency has a similar effect on the stability of another mRNA containing a class II ARE, i.e., the mRNA encoding GM-CSF. In particular, there was a marked stabilization of GM-CSF mRNA in bone marrow stromal cells derived from TTP-deficient mice, compared to control cells, indicating  
25       that TTP is also a physiological regulator of GM-CSF mRNA stability, and thus, of GM-CSF secretion.

Recombinant GM-CSF has been administered to human patients for a variety of indications, including the bone marrow suppression that accompanies certain forms of  
30       chemotherapy, autologous bone marrow transplantation, aplastic anemia, and other

neutropenic conditions. The present studies allow the development of new therapeutic approaches for stimulating GM-CSF production, for example, in a patient or subject, by increasing the stability of its mRNA.

5           Accordingly, a method of treating granulocytopenia in a subject is provided. The method comprises administering to the subject an agent that inhibits the degradation of GM-CSF mRNA. The granulocytopenia may be relative granulocytopenia (reduction in granulocyte count below a level considered to be clinically normal) or absolute granulocytopenia (the absence of granulocytes). Treating  
10 granulocytopenia involves an increase in granulocyte count to a normal count or approaching a normal count, or it can involve an increase in granulocyte count that is significant compared to the granulocyte count prior to treatment with the agent. The determination that treatment has occurred can be based on clinical parameters such as those applied by a physician in medical practice or the determination can be based on  
15 the measurement of a subclinical parameter that is shown to be a reasonable indicator of clinical improvement.

The patient or subject can be a human or non-human primate, or any animal that experiences granulocytopenia (e.g., a cat, a dog, a horse, a bird, or a rodent) as part of a  
20 pathological condition or exposure to a granulocyte-depleting amount of a toxic substance (e.g., a chemotherapeutic agent). Additionally, populations of cells *in vitro* can be enriched for granulocytes according to the present method. These cells may be from cell culture or they may be primary cells *ex vivo*. These populations of cells can be used as research tools to study GM-CSF or they can be returned to the subject.

25

The methods of the invention can be used to treat granulocytopenia of whatever cause, particularly diseases that result from a shortage of granulocytes, by increasing the level of GM-CSF in the treated subject. Diseases that are caused by a shortage of granulocytes include granulocytopenia generally, and, specifically, granulocytopenia  
30 associated with cancer chemotherapy; associated with propylthiouracil use; associated

with other drug use besides chemotherapeutic agents and propylthiouracil; associated with radiotherapy for marrow ablation for bone marrow transplantation or for other conditions; primary granulocytopenia; aplastic anemia; myelofibrosis and myeloid metaplasia; systemic lupus erythematosus; congenital neutropenia, chronic neutropenic disease, cyclic neutropenia, AIDS, myelodysplastic syndromes, myeloid leukemia, acute myeloid leukemia, other forms of myeloablative treatment. Examples of the clinical parameters that can be used to measure the status of the disease (e.g., treatment) include granulocyte counts, as well as measurement of peripheral blood granulocyte precursors, e.g. band forms, and marrow aspiration or marrow biopsy for myeloid lineage cells. Examples of these methods are well known in the art.

Increased levels of GM-CSF are provided by inhibiting the degradation of GM-CSF mRNA. This is accomplished by inhibiting the mRNA degradative activity of certain proteins identified herein as having GM-CSF mRNA degradative activity.

15

Herein it is shown that tristetraprolin (TTP) stimulates degradation of GM-CSF mRNA (see, e.g., Examples 1 and 4). Without being bound by theory, the mRNA degradative activity of TTP is likely to be a function of its ability to recruit a deadenylating enzyme into proximity with the GM-CSF mRNA. Thus, an agent that inhibits the degradation of GM-CSF mRNA can be an agent that inhibits the mRNA degradative activity of TTP, for example, a competitor of TTP. A competitor of TTP can compete with TTP for binding to the AU-rich element (ARE) of GM-CSF mRNA, thereby partially or completely inhibiting the binding of TTP (or a TTP-like protein) to the AU-rich element. Alternatively, a competitor of TTP can compete with TTP for binding to an mRNA degradative enzyme (e.g., a deadenylase, exonuclease (e.g., a 3' exonuclease) or endonuclease) that plays a role in TTP-induced GM-CSF mRNA degradation. Examples of the agents that inhibit TTP induced mRNA degradation include certain mutant TTP molecules described herein. Other agents, such as chelators of zinc, can also inhibit TTP's mRNA degradative activity.

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As shown herein, members of the ERF1 and ERF2 families of TTP-like proteins also stimulate degradation of TNF- $\alpha$  and GM-CSF mRNA. ERF1 and ERF2 are TTP-like proteins of the CCCH double zinc finger class. There is weak similarity between TTP and ERF1 or ERF2 in the non-zinc finger domains, but they are very similar (highly conserved) in the zinc finger domains, both having a lead-in sequence of R(K)YKTEL, and then two zinc fingers, spaced 18 amino acids apart, that each have the composition C<sub>x</sub>GxC<sub>x</sub>YG<sub>x</sub>(K/R)C<sub>x</sub>F<sub>x</sub>H, where x represents various amino acids. The cloning of ERF1 and the alignment of human and rat ERF1 are described in Barnard et al., *Nucleic Acids Res.* 21:3580, 1993, and the cloning of ERF2 is described in Nie et al., *Gene* 152:285-286, 1995. ERF1 and ERF2 function analogously to TTP, and therefore, competitors and inhibitors of ER! and ERF2 may be identified as described for TTP above.

The 64 amino acid double zinc finger domain of TTP and TTP-related polypeptides (see Fig. 5B) is sufficient to accomplish all of the relevant functions of TTP, i.e. stimulating degradation of TNF $\alpha$  mRNA, interleukin 3 (IL3) mRNA and GM-CSF mRNA in transfection studies in intact cells (see Example 3 for these protocols); binding to the AREs of these three mRNAs in cell-free crosslinking studies; and binding to the AREs in cell-free gel shift studies. The two TTP related proteins, represented by their rat and *Xenopus laevis* homologues, respectively, have the same effect as TTP on all three mRNAs in all three assays. Alignment of the human and *Xenopus* versions of ERF2 are provided in De et al. (Identification of four CCCH zinc finger proteins in *Xenopus*, including a novel vertebrate protein with four zinc fingers and severely restricted expression, *Gene* 228:133-145, 1999).

25

Therefore, all three known members of this CCCH double zinc finger class can be used in the present methods, as can the subdomains of the three proteins that contain the double zinc finger domain, and thus exhibit all the activities of TTP. Examples of other such proteins that are identified according to the methods taught herein can also be used in the ways taught for TTP, ERF1, and ERF2. For example, a fourth member

30

of the class, XC3H-4 (De et al., *Gene* 228:133-145, 1999), has recently been identified, which contains the double zinc finger domain and appears to possess similar activity to the other members of this class. The relevant protocols for the study of ERF, ERF2 and fragments of TTP are those described in Example 3, except that ERF1 and ERF2, as  
5 well as fragments of TTP, were used in place of the full length TTP used in that example.

Further examples of proteins that can act as competitors of TTP for ARE binding, include ERF1 and ERF2 as described above; XC3H-4 and its mammalian  
10 putative mammalian homologue; TIAR and TIA-1, the AUF-1 family, HuR, HuC, Hel-N1, HuD, AU-B. In fact, ERF1, ERF2 and XC3H-4 do compete with TTP for ARE binding, but since they have TTP-like activities, they would be less effective in protocols to decrease TTP-induced mRNA degradation. The other proteins in the above list are not expected to have TTP-like activities, and are expected to have an  
15 ARE- protective effect due to competition with TTP, ERF1, ERF2 etc. Mutants of TTP, ERF1, ERF2, etc. that do not bind the ARE could still inhibit the activities of wild type TTP, ERF1, ERF2 etc. by interacting with degradative enzymes such as the deadenylases or exonucleases.

20 A method of treating granulocytopenia in a subject is provided, comprising administering to the subject a mutant TTP that has reduced activity compared to wild type TTP. This method works because the mutant competes with wild type TTP expressed in the cell. The amount of mutant expressed can be measured and its sufficiency to compete with wildtype TTP can be assessed routinely using the methods  
25 taught herein. The activity of TTP reduced is degradation of GM-CSF mRNA. The mutant TTP can be administered directly or it can be administered by delivering to the subject a nucleic acid that expresses the mutant TTP in cells of the subject.

Another method of treating granulocytopenia in a subject comprises  
30 administering to the subject a mutant ERF1 that has reduced activity compared to wild

type ERF1. This method works because the mutant competes with wild type ERF1 or TTP expressed in the cell. The amount of mutant expressed can be measured and its sufficiency to compete with wild type ERF1 can be assessed routinely using the methods taught herein. The activity of ERF1 reduced is degradation of GM-CSF mRNA. The mutant ERF1 can be administered directly or it can be administered by delivering to the subject a nucleic acid that expresses the mutant ERF1 in cells of the subject.

Another method of treating granulocytopenia in a subject comprises administering to the subject a mutant ERF2 that has reduced activity compared to wild type ERF2. This method works because the mutant competes with wild type ERF2 or TTP expressed in the cell. The amount of mutant expressed can be measured and its sufficiency to compete with wild type ERF2 can be assessed routinely using the methods taught herein. The activity of ERF2 reduced is degradation of GM-CSF mRNA. The mutant ERF2 can be administered directly or it can be administered by delivering to the subject a nucleic acid that expresses the mutant ERF2 in cells of the subject.

A mutant TTP that has a reduced TTP activity compared to wild type TTP is provided. In the mutant TTP, the activity of TTP reduced is TTP binding to the ARE of GM-CSF mRNA. Alternatively, in the mutant TTP, the activity of TTP reduced is TTP binding to an mRNA degradative enzyme. The activity of TTP reduced is degradation of GM-CSF mRNA, although the actual activity of the mutant can take place at any point in the degradative pathway. A mutant with this activity is also referred to as a dominant negative mutant.

Examples of the TTP mutant that reduce GM-CSF mRNA degradation include C124R and C147R (coordinates based on the numbering system disclosed in Genbank accession number AAA61240 for human TTP). All the numbering for the TTP mutants describe herein is based on this sequence. These mutants and their activities are

described in Example 3. Additional dominant negative mutants of TTP are provided. They include (using the same numbering system) H128K; H166L; F126N; deletion of F164; and insertion of Q after I165. All are non-binding mutants that exhibit dominant negative activity in assays such as those taught in Example 3.

5

A mutant ERF1 that has a reduced ERF1 activity compared to wild type ERF1 is provided. In the mutant ERF1, the activity of ERF1 reduced is ERF1 binding to the ARE of GM-CSF mRNA. Alternatively, in the mutant ERF1, the activity of ERF1 reduced is ERF1 binding to an mRNA degradative enzyme. The activity of ERF1 reduced is degradation of GM-CSF mRNA, although the actual activity of the mutant can take place at any point in the degradative pathway. A mutant with this activity is also referred to as a dominant negative mutant.

10

Examples of mutations in the rat version of ERF1 that are the equivalent of C124R and C147 R of TTP have also been made. The equivalent mutations to human ERF1 are C135R and C158R based on Genbank accession number NP\_004917. These are also non-binding and dominant negative mutants as tested in protocols essentially like those described in Example 3, except using the ERF1 mutants rather than TTP.

15

A mutant ERF2 that has a reduced ERF2 activity compared to wild type ERF2 is provided. In the mutant ERF2, the activity of ERF2 reduced is ERF2 binding to the ARE of GM-CSF mRNA. Alternatively, in the mutant ERF2, the activity of ERF2 reduced is ERF2 binding to an mRNA degradative enzyme. The activity of ERF2 reduced is degradation of GM-CSF mRNA, although the actual activity of the mutant can take place at any point in the degradative pathway. A mutant with this activity is also referred to as a dominant negative mutant.

20

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Active fragments of the TTP, ERF1, ERF2, XC3H-4 and other TTP-like proteins are provided. The fragments can possess the mRNA degradative activity of the full-length molecule or they can compete with the full-length molecule for binding

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to the ARE, and, thus, inhibit the degradative activity of the full-length molecule. Similarly, fragments of the mutants of these proteins are contemplated. Whether the fragment has a degradative activity or inhibits degradation can be routinely determined using the methods taught herein.

5

The nucleic acids of this invention (e.g., those encoding wild-type or mutant TTP or TTP-like proteins or fragments thereof) can be administered using *in vivo* gene therapy techniques (US Patent No. 5,399,346). With regard to gene therapy applications, the nucleic acid can comprise a nucleotide sequence which encodes a gene product which is meant to function in the place of a defective gene product and restore normal function to a cell which functioned abnormally due to the defective gene product. Alternatively, the nucleic acid may encode a gene product which was not previously present in a cell or was not previously present in the cell at a therapeutic concentration, whereby the presence of the exogenous gene product or increased concentration of the exogenous gene product imparts a therapeutic benefit to the cell and/or to a subject.

For *in vivo* administration, the cells can be in a subject and the nucleic acid can be administered in a pharmaceutically acceptable carrier. The subject can be any animal in which it is desirable to selectively express a nucleic acid in a cell. In a preferred embodiment, the animal of the present invention is a human. In addition, non-human animals which can be treated by the method of this invention can include, but are not limited to, non-human primates, cats, dogs, birds, horses, cows, goats, sheep, guinea pigs, hamsters, gerbils and rabbits, as well as any other animal in which selective expression of a nucleic acid in a cell can be carried out according to the methods described herein.

In the method described above which includes the introduction of exogenous DNA into the cells of a subject (i.e., gene transduction or transfection), the nucleic acids of the present invention can be in the form of naked DNA or the nucleic acids can

be in a vector for delivering the nucleic acids to the cells for expression of the nucleic acid inside the cell. The vector can be a commercially available preparation, such as an adenovirus vector (Quantum Biotechnologies, Inc. (Laval, Quebec, Canada). Delivery of the nucleic acid or vector to cells can be via a variety of mechanisms. As one

5 example, delivery can be via a liposome, using commercially available liposome preparations such as Lipofectin®, Lipofectamine® (GIBCO-BRL, Inc., Gaithersburg, MD), Superfect® (Qiagen, Inc. Hilden, Germany) and Transfectam® (Promega Biotec, Inc., Madison, WI), as well as other liposomes developed according to procedures standard in the art. In addition, the nucleic acid or vector of this invention can be

10 delivered *in vivo* by electroporation, the technology for which is available from Genetronics, Inc. (San Diego, CA) as well as by means of a Sonoporation machine (ImaRx Pharmaceutical Corp., Tucson, AZ).

As one example, vector delivery can be via a viral system, such as a retroviral

15 vector system which can package a recombinant retroviral genome. The recombinant retrovirus can then be used to infect and thereby deliver nucleic acid to the infected cells. The exact method of introducing the nucleic acid into mammalian cells is, of course, not limited to the use of retroviral vectors. Other techniques are widely available for this procedure including the use of adenoviral vectors, adeno-associated

20 viral (AAV) vectors, lentiviral vectors, pseudotyped retroviral vectors, and pox virus vectors, such as vaccinia virus vectors. Physical transduction techniques can also be used, such as liposome delivery and receptor-mediated and other endocytosis mechanism. This invention can be used in conjunction with any of these or other commonly used gene transfer methods.

25

The nucleic acid and the nucleic acid delivery vehicles of this invention, (e.g., viruses; liposomes, plasmids, vectors) can be in a pharmaceutically acceptable carrier for *in vivo* administration to a subject. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be

30 administered to a subject, along with the nucleic acid or vehicle, without causing any

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undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one  
5 of skill in the art.

The nucleic acid or vehicle may be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, transdermally, extracorporeally, topically or the like. The exact amount of the nucleic acid or vector  
10 required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity or mechanism of any disorder being treated, the particular nucleic acid or vehicle used, its mode of administration and the like.

The compounds of this invention (e.g., nucleic acids, proteins, polypeptides, small molecules) can be administered to a cell of a subject either *in vivo* or *ex vivo*. For administration to a cell of the subject *in vivo*, as well as for administration to the subject, the compounds of this invention can be administered orally, parenterally (e.g., intravenously), by intramuscular injection, by intraperitoneal injection, subcutaneous  
15 injection, transdermally, extracorporeally, topically, mucosally or the like.  
20

Depending on the intended mode of administration, the compounds of the present invention can be in pharmaceutical compositions in the form of solid, semi-solid or liquid dosage forms, such as, for example, tablets, suppositories, pills, capsules,  
25 powders, liquids, suspensions, lotions, creams, gels, or the like, preferably in unit dosage form suitable for single administration of a precise dosage. The compositions will include, as noted above, an effective amount of the selected composition, possibly in combination with a pharmaceutically acceptable carrier and, in addition, may include other medicinal agents, pharmaceutical agents, carriers, adjuvants, diluents, etc.

Parenteral administration of the compounds of the present invention, if used, is generally characterized by injection. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. As used herein, "parenteral administration" includes intradermal, subcutaneous, intramuscular, intraperitoneal, intravenous and intratracheal routes. A more recently revised approach for parenteral administration involves use of a slow release or sustained release system such that a constant dosage is maintained. See, e.g., U.S. Patent No. 3,610,795, which is incorporated by reference herein. These compounds can be present in a pharmaceutically acceptable carrier, which can also include a suitable adjuvant. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the selected compound without causing substantial deleterious biological effects or interacting in a deleterious manner with any of the other components of the composition in which it is contained.

The exact amount of the compound required will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the particular compound used, its mode of administration and the like. Thus, it is not possible to specify an exact amount for every compound. However, an appropriate amount can be determined by one of ordinary skill in the art using only routine experimentation given the teachings herein.

If *ex vivo* methods are employed, cells or tissues can be removed and maintained outside the subject's body according to standard protocols well known in the art. The compounds of this invention can be introduced into the cells via known mechanisms for uptake of small molecules into cells (e.g., phagocytosis, pulsing onto class I MHC-expressing cells, liposomes, etc.). The compounds of this invention can also be linked to the homeodomain of Antennapedia for introduction, i.e. internalization of the compound, into cells (*Prochiantz, P.* "Getting hydrophilic compound into cells: lessons from homeopeptides", *Curr. Opin. Neurobiol.* 6(5): 629-634.) The cells can

then be infused (e.g., in a pharmaceutically acceptable carrier) or transplanted back into the subject per standard methods for the cell or tissue type. Standard methods are known for transplantation or infusion of various cells into a subject.

- 5           The invention provides methods of screening an agent for the ability to inhibit an activity of TTP or an activity of TTP-like proteins (e.g., ERF1, ERF2, and XC3H-4). For example, a method of screening an agent for the ability to inhibit an activity of TTP is provided. The method can comprise the steps of: a) co-transfecting a cell with a nucleic acid that encodes TTP and a nucleic acid that comprises a TTP binding
- 10   sequence (e.g., an ARE) downstream of a nucleic acid sequence encoding a reporter protein; b) contacting the cell of step a) with the agent; and c) comparing the expression of the reporter protein in the cell of step b) to the cell of step a) in the absence of the agent, a reduction of expression in the cells of step b) compared to the cells of step a) indicating that the agent has the ability to inhibit an activity of TTP.
- 15   Examples of this type of assay are described in Example 2 and Example 3. The steps of the above method can be applied to determine whether an agent can inhibit ERF1 by substituting an ERF1 encoding nucleic acid for a TTP encoding nucleic acid, and by substituting a nucleic acid that contains an ERF1 binding site for the nucleic acid that contains a TTP binding site. Similarly, the method can be used to determine whether
- 20   an agent can inhibit ERF2, by substituting any ERF2 encoding nucleic acid and nucleic acid with and ERF2 binding sequence for the TTP sequences described above.

- A method of screening an agent for the ability to compete with TTP or TTP-like protein (e.g., ERF1, ERF2, XC3H-4, etc.) for binding to the ARE of mRNA is
- 25   provided. For example, the method can comprise the steps of: a) transfecting a cell with a nucleic acid that encodes an epitope-tagged TTP or untagged TTP in the cell; b) obtaining a cytosolic extract of the cells of step a); c) contacting the cytosolic extract of step b) with the agent or an appropriate control; d) contacting the cytosolic extract of steps b) and c) with a probe from the 3' UTR of an mRNA; e) comparing the binding
- 30   of the probe to the TTP in the cytosolic extract of step b) with the binding of the the

probe to the TTP in the cytosolic extract of step c), the presence of reduced binding of the probe to TTP in the cytosolic extract of step c) indicating an agent that can compete with TTP for binding to the ARE of mRNA. TTP binding to ARE probes can be readily demonstrated in cell-free assays, making possible screens for potential inhibitors of this interaction. These steps can be adapted to detect competitors of ERF1, ERF2, XC3H-4 etc. by substituting these molecules for TTP.

A variety of assay methods can be used to determine whether a given compound interferes with TTP or related protein binding to the GM-CSF ARE and the breakdown of GM-CSF mRNA. These would include cell-based experiments, such as the transfection studies in 293 cells cited in Example 3; it can be seen that addition of cell-permeable compounds to the cells that inhibited the TTP-mRNA interaction would result in inhibition of TTP's ability to deadenylate and destroy the mRNA. Such assays could use a variety of more convenient readouts, e.g. luminescent proteins, human growth hormone, chloramphenicol acetyltransferase, beta-galactosidase, etc. Similar cell based studies could also be performed in yeast, where there is considerable precedent for high-throughput screening assays for protein interactions with DNA, RNA and other proteins. Cell-free assays would probably be the most convenient to set up; these would involve extracts from cells expressing TTP or its related proteins (e.g., ERF1, ERF2, etc.) or its active fragments (e.g., the double zinc finger domain), and testing their ability to bind to purified, labeled GM-CSF ARE, assayed by either crosslinking or gel-shift assays as described in the Examples. More conveniently still, these assays could use purified TTP or its active fragments, or purified members of the TTP-related protein class or their active fragments, or fusion proteins expressing TTP or its related proteins or their fragments. All have been shown to be active at binding and crosslinking to the TNF $\alpha$  ARE. These would use variable lengths of sequence of the CM-CSF ARE – e.g., a probe that corresponds to bases 3390 – 3467 of Genbank accession number X03020, but the experiments with the TNF ARE have shown that this could probably be shortened to a “core” ARE of about 23 bases (bases 1309 to 1332 of Genbank Accession number X02611 and corresponding bases for GM-CSF).

- It is also possible to use other assays that interfere with TTP's putative interaction with a deadenylase. Such an enzyme has been recently cloned (Korner CG. Wormington M. Muckenthaler M. Schneider S. Dehlin E. Wahle E. "The deadenylating nuclease (DAN) is involved in poly(A) tail removal during the meiotic maturation of
- 5 Xenopus oocytes "EMBO J. 17, 5427-5437 (1998)), and could be used in cell-free assays. Another recently devised assay (Ford LP. Watson J. Keene JD. Wilusz J. ELAV proteins stabilize deadenylated intermediates in a novel in vitro mRNA deadenylation/degradation system. Genes & Development. 13:188-201, 1999) can be used that uses whole cell extracts to look at deadenylation of the TNF mRNA.
- 10 Stimulatory effects of TTP on this reaction, and inhibitory effects of the dominant negative mutants are, thus, determined.

#### Test Compounds

- In general, compounds that modulate the activity of TTP and TTP-like
- 15 polypeptides may be identified from large libraries of natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the screening procedure(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds
- 20 can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any
- 25 number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, polypeptide- and nucleic acid-based compounds. Synthetic compound libraries are commercially available, e.g., from Brandon Associates (Merrimack, NH) and Aldrich Chemical (Milwaukee, WI).

Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, e.g., Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographics Institute (Ft. Pierce, FL), and PharmaMar, U.S.A. (Cambridge, MA). In addition, natural and  
5 synthetically produced libraries are generated, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

10 In addition, those skilled in the art of drug discovery and development readily understand that methods for dereplication (e.g., taxonomic dereplication, biological dereplication, and chemical dereplication, or any combination thereof) or the elimination of replicates or repeats of materials already known for their effect on the activity of TTP or a TTP-like polypeptide should be employed whenever possible.

15 When a crude extract is found to have a desired activity, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification process is the careful characterization and identification of a chemical entity within the crude  
20 extract having an activity that stimulates or inhibits a particular target TTP or TTP-like polypeptide. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for  
25 treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic value may be subsequently analyzed using animal models for diseases or conditions in which it is desirable to regulate or mimic activity of TTP or a TTP-like polypeptide.



The present invention is more particularly described in the following examples, which are intended only to be illustrative, since numerous modifications and variations therein will be apparent to those of ordinary skill in the art.

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**EXAMPLE 1****TTP is a Regulator of GM-CSF mRNA Deadenylation and Stability****Methods**

10

*Mice.*

Mice deficient in TTP were generated in our laboratory by interbreeding heterozygous animals as described (12). Genotyping of the offspring was performed by PCR of tail DNA, using a set of primers that span the region of the wild-type gene that was disrupted by the targeting vector, and another set that amplified a fragment of the Neo-cassette inserted in the targeting vector (12). Mice deficient in both TNF $\alpha$  receptors (TNFR) (17, 18) were kindly provided by Dr. Mark W. Moore (Genentech, South San Francisco, CA), and were interbred with the TTP heterozygous animals. Genotyping of the offspring was performed by PCR of tail DNA, using primers that span the region of the wild-type genes disrupted by the targeting vectors. Genotyping was also performed using Southern blotting of tail DNA after digestion with *Bgl*II and probing for Neo, with a 0.7 kb fragment of the vector PMC1neoPolyA (Stratagene, LaJolla, CA); this technique revealed three bands of ~3.5 kb (TNFR1), ~2.5 kb (TTP) and ~2 kb (TNFR2). Triple-heterozygous mice were interbred to yield triple-homozygous offspring. As controls for these animals, animals deficient in both TNFR but containing both TTP alleles were generated. All animals were maintained in autoclaved microisolator cages in a barrier facility. Animal care and all experiments were in accordance with institutional guidelines for animal use.

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*Culture of bone marrow stromal cells.*

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Primary cultures of bone marrow stromal cells were established according to the

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protocol described by Dexter et al. (19, 20) and modified by Van Den Heuvel et al. (21). Briefly, marrow cells from both femurs were flushed with minimum essential medium alpha ( $\alpha$ -MEM) using a 1 ml syringe attached to a 25G needle. After centrifugation for 5 min at 4°C at 500g, the cells were resuspended in 0.15 M ammonium chloride to lyse the red cells. After a 5 min incubation at room temperature, the cells were re-centrifuged as above, and then resuspended in culture medium ( $\alpha$ -MEM supplemented with 25 % (vol/vol) fetal calf serum (GIBCO BRL, Gaithersburg, MD), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM glutamine, and 1.25  $\mu$ g/ml fungizone (all additives from GIBCO BRL)). Cells were plated onto 60-100 mm diameter petri dishes, as many plates as required for a given experiment (normally, 5-12 plates/animal), and cultured at 33°C in a humidified atmosphere containing 5% CO<sub>2</sub> for 4 days. After that, the medium was replaced by fresh culture medium and then replaced at weekly intervals until confluence was achieved (usually 4-6 weeks).

15

For identification of cell types, cells were trypsinized (0.05% trypsin (wt/vol)/0.53 mM EDTA, GIBCO BRL) and replated in the same culture medium at 50,000 cells/well in 4-well Lab-Tek tissue culture chambers (Nunc, Thousand Oaks, CA), and incubated for another 48 hrs before performing any of the stains or assays. Morphology was assessed by staining the cells with the Diff-Quick Stain Set (Baxter Healthcare Corporation, McGaw Park, IL). Non-specific esterase staining was performed as described (22), using  $\alpha$ -naphthyl acetate as a substrate (Sigma Chemical Co., St. Louis, MO). Phagocytosis of latex beads was performed for 30 min as previously described (23), using 0.8  $\mu$ m latex beads (Sigma). Oil red O stain was used to identify fat cells. Cells were analyzed and photographed using a Nikon Eclipse 400 microscope (Southern Micro Instruments, Atlanta, GA), equipped with an Olympus PM-C35B camera (Olympus America Inc., Lake Success, NY). Uptake of Dil-acetylated LDL (Biomedical Technologies, Inc., Stoughton, MA) was used to identify macrophages and endothelial cells, and was performed as described by Agui et al. (24). Cells were analyzed using a Nikon Eclipse 600 microscope (Southern Micro

Instruments). At least 500 cells per genotype, in duplicate, were counted in each assay.

#### *Northern blotting*

When indicated, the cells were stimulated with lipopolysaccharide (LPS) (1  
5  $\mu\text{g/ml}$ ) (Sigma) or mouse recombinant  $\text{TNF}\alpha$  (10  $\text{ng/ml}$ ) (R & D Systems, Inc.,  
Minneapolis, MN) for different times, and RNA was extracted with the RNeasy kit  
from Qiagen, Inc. (Valencia, CA), according to the directions provided by the  
manufacturer. RNA was analyzed by Northern blot as described (25), except that the  
10 gels contained 1.5% (w/v) agarose. Filters were sequentially probed with cDNA probes  
to mouse GM-CSF (plasmid p5'mGM-CSF, containing the sequences of exons I, II, III  
and part of the exon IV of the mouse GM-CSF (bp 1136-1317, 1415-1456, 2214-2339,  
3082-3153 of GenBank accession number X03020), was constructed by RT-PCR using  
RNA from Raw 264.7 cells treated for 4 hrs. with 1  $\mu\text{g/ml}$  of LPS as the template for  
RT. The 5' primer for PCR amplification was  
15 5'gtcgacACTCAGAGAGAAAGGCTAAGG<sup>3'</sup>, and the 3' primer was  
5'CATTCAAAGGGgatatcAGTCAG<sup>3'</sup>, where the lower case letters indicate the  
restriction sites for SalI and EcoRV, respectively (the EcoRV site is a naturally  
occurring site in the mGM-CSF gene). The resulting PCR product was digested with  
these enzymes and cloned into the SalI, EcoRV and XbaI sites of the vector pSK-  
20 (Stratagene). Correct sequence of the plasmid insert was confirmed by dye terminator  
cycle sequencing (Perkin-Elmer, Foster City, CA)) and rat GAPDH (26). The 423 bp  
SalI-EcoRV insert from the GM-CSF and the 1.3 kb EcoRI insert from the GAPDH  
cDNAs were isolated from low-melting point agarose gels and random primer labeled  
with  $\alpha$ -<sup>32</sup>P dCTP for Northern hybridization.

25

In the RNA stability experiments, BMSC were cultured in the presence of 1  
 $\mu\text{g/ml}$  LPS for 2 hrs, after which the LPS-containing medium was removed and  
replaced by fresh medium containing 5  $\mu\text{g/ml}$  of actinomycin D (Sigma). Cells were  
then harvested for the preparation of RNA at 15 min intervals, using the Qiagen  
30 RNeasy kit as described above. Analysis of Northern blots for  $\text{TNF}\alpha$  and GAPDH

mRNA was performed using PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA); in the case of the GM-CSF mRNA, laser-scanning densitometry was performed using a Zeineh soft laser scanning densitometer (model SL-504-XL, Biomed Instruments Inc., Fullerton, CA). This was attempted only when at least one of the  
5 peak areas was  $\geq 20$  arbitrary densitometry units. RNase H assays were performed as described (16).

#### *Measurement of GM-CSF secretion*

To assess GM-CSF secretion, BMSC were cultured in 24-well plates for 6  
10 weeks, then stimulated with LPS (1  $\mu$ g/ml) for 24 hrs, after which the supernatants were removed and stored at -80°C until used. GM-CSF secretion was assessed by ELISA, using a specific kit for mouse GM-CSF from Endogen (Woburn, MA), following the specifications of the manufacturer.

15

#### **Results**

##### *Characteristics of bone marrow stromal cells (BMSC) from wild type and TTP-deficient mice.*

20 Although we were not able to detect GM-CSF mRNA expression in primary macrophages by Northern blotting, our previous data suggested that BMSC might be involved in the development of the TTP-deficiency phenotype (13). BMSC are a mixture of fibroblast-like, macrophage-like, endothelial cells and adipocytes that provide the microenvironment and growth factors needed for the normal development  
25 of the hematopoietic system (19, 27).

We first examined primary BMSC cultures from wild type (WT) and TTP-deficient mice after 4-6 weeks of culture at 33°C. These conditions maintain the cultures in a non-hematopoietic state, i.e., hematopoietic progenitors do not survive the  
30 first stages of the culture, leaving behind purely stromal cells that are still capable of

producing hematopoietic growth factors (21). This point was confirmed by the absence of hematopoietic precursors or mature polymorphonuclear cells in the stained cultures. To evaluate the relative proportions of each cell type in the cultures, we performed a series of specific assays. Dil-acetylated LDL is avidly taken up by macrophages and endothelial cells, which can be then differentiated by morphology (24). Using this assay, WT cultures contained 64% positive cells, while TTP-deficient cultures contained 60% positive cells. The negative cells in these cultures are considered to be fibroblast-like cells (24). Non-specific esterase is a selective cytochemical stain for macrophages (22). Using this method, 47% cells were positive in the WT cultures and 48% in the TTP-deficient cultures. Macrophages are also highly phagocytic for latex beads. In these cultures, 41% of the WT cells and 39% of the TTP-deficient cells were phagocytic for latex beads. The use of oil red O stain, specific for fat cells, revealed that fewer than 1% of cells were fat cells in both WT or TTP-deficient cultures (24, 28). Taken together, these results indicate that the relative proportions of each cell type were comparable between WT and TTP-deficient cultures. Thus, the differences observed between the two genotypes with respect to GM-CSF production were not likely to be due to differences in the proportions of different cell types in the cultures.

*Expression of GM-CSF mRNA in bone marrow stromal cells from WT and TTP-deficient mice.*

After the BMSC became confluent, they were stimulated with either LPS (1µg/ml) or TNFα (10 ng/ml), and the effect of these factors on GM-CSF mRNA accumulation was studied over a period of 8 hrs. When comparing wild type (WT) and knockout (KO) samples or heterozygous (Htz) and KO samples, identical amounts of total cellular RNA were subjected to electrophoresis in parallel gels; blotting was performed in parallel; and both were hybridized together with the same probe and exposed to film in the same autoradiography cassette. LPS induced detectable levels of GM-CSF mRNA in WT cells within 2 hrs; these levels peaked at 3 hrs and then slowly decreased over the next several hours, with a slight increase at 8 hrs. GM-CSF mRNA does not appear as a single species in these cells, but as two major components of

approximately 1.0 and 0.8 kb, as previously described (29). When an identical study was performed with cells derived from the TTP-deficient mice, however, there were several differences in the pattern of GM-CSF mRNA expression. First, the GM-CSF mRNA was detectable earlier in the TTP-deficient cells (after 1 hr of exposure to LPS);

5 second, the overall accumulation of mRNA at the peak time (3 hrs) appeared to be approximately two-fold greater in the TTP-deficient cells than in the WT cells (after normalizing for GAPDH mRNA expression); and third, the distribution of the two major mRNA species was different. In the WT cells, the average proportion of the lower band, expressed as percentage of the total GM-CSF mRNA, was  $40 \pm 3\%$  (mean

10  $\pm$  S.E.M. of 7 values); in contrast, the lower band from the TTP-deficient cells contained only  $16 \pm 1\%$  (mean  $\pm$  S.E.M. of 8 values) of the total ( $p < 0.0001$  when compared to WT values by Student's t test).

When  $\text{TNF}\alpha$  was used as the stimulus, these differences were even more

15 pronounced. The total amount of GM-CSF mRNA accumulation was much greater (approximately 5-fold at the time of the greatest difference, i.e., 5 hrs) than that seen in the control cells (in this case, derived from a heterozygous (Htz) mouse). In addition, GM-CSF mRNA expression was detected earlier (45 min) and remained detectable at identical autoradiographic exposures for a much longer period (up to 8 hrs in the KO,

20 versus 5 hrs in the Htz cells). Finally, almost no smaller form of GM-CSF mRNA was detectable in the TTP-deficient cells ( $46 \pm 4\%$  in the Htz ( $n = 7$ ) versus  $11 \pm 2\%$  ( $n = 9$ ) in the KO cells,  $p < 0.0001$ ).

*GM-CSF mRNA adenylation state in BMSC from WT and TTP-deficient mice.*

25 Because TTP has been shown to promote the deadenylation of a synthetic modified  $\text{TNF}\alpha$  mRNA in a co-transfection system (16), we determined whether the smaller GM-CSF mRNA species that was prominent in the WT and Htz cells but nearly absent in the TTP-deficient cells was the deadenylated form of the GM-CSF mRNA. RNase H treatment on selected samples of GM-CSF mRNA. After hybridization with

30 oligo (dT) and digestion with RNase H to remove any remaining polyA tails, the RNA

was analyzed by Northern blot. The addition of RNase H completely eliminated the larger species of GM-CSF mRNA, leaving only a single form that co-migrated exactly with the smaller band present in the Htz cells but almost absent in the TTP-deficient cells. This result establishes that the two major forms of GM-CSF mRNA observed in the WT and Htz cells, as well as in earlier studies (29), correspond to the polyadenylated and deadenylated forms of GM-CSF mRNA, respectively, and that the absence of TTP results in a marked increase in the proportion of the polyadenylated form relative to the deadenylated form. By comparing the migration position of the two bands with RNAs of known size, we estimate that the polyA tail of the fully polyadenylated form was approximately 220 residues long in these cells.

*Half-life of GM-CSF mRNA in BMSC from WT and TTP-deficient mice.*

Because deadenylation is a process associated with the initiation of mRNA degradation (30), we next evaluated the half-life of GM-CSF mRNA in BMSC from WT and TTP-deficient mice. After incubation of BMSC with LPS for 2 hrs followed by the addition of actinomycin D (5 µg/ml), there was a gradual disappearance of GM-CSF mRNA from the WT cells, with an estimated half-life of 111 min. In contrast, in the TTP-deficient cells there was essentially no disappearance of the GM-CSF mRNA over the 60 min period of actinomycin D treatment, with no calculable half-life. These data confirm that the absence of TTP results in an increase in the stability of GM-CSF mRNA. As in the previous experiments, the proportion of GM-CSF mRNA in the smaller, deadenylated form was much greater ( $32 \pm 2\%$ ) in the WT ( $n=5$ ) than in the TTP-deficient cells ( $8 \pm 0.3\%$ ) ( $n=5$ ) ( $p<0.0001$ ). Although we cannot exclude effects of TTP-deficiency on transcription of the GM-CSF gene, these results demonstrate that both GM-CSF mRNA stability and the proportion of the message in the fully polyadenylated form are increased in the BMSC from the TTP-deficient mice.

To test for the specificity of this effect, we probed the same filters with cDNAs for both TNFα and c-fos. There was increased stability of TNFα mRNA in the TTP-deficient BMSC, confirming our previous results in macrophages (11); in the BMSC,

the calculated half-life for TNF $\alpha$  mRNA in the WT cells was 35 min, versus 90 min in the TTP-deficient cells. There was also the apparent absence of a stable, deadenylated intermediate form of the TNF $\alpha$  mRNA, in contrast to the results with GM-CSF mRNA. When similar studies were performed with c-fos mRNA, another short-lived mRNA  
5 that contains a so-called class I ARE (31), the estimated half-life of the mRNA was 43 min in the WT cells versus 41 min in the TTP-deficient cells. These results suggest but do not prove that the TTP effect is specific to a particular set of mRNAs, those containing class II AREs.

10      *Secretion of GM-CSF from BMSC from WT and TTP-deficient mice.*

Our previous results with TNF $\alpha$  showed that the increased stability of TNF $\alpha$  mRNA was accompanied by increased secretion of the protein (13). To determine whether a similar situation occurred with GM-CSF, we incubated confluent layers of BMSC in the presence of LPS (1  $\mu$ g/ml) for 24 hrs, and measured the amount of GM-  
15 CSF secreted into the culture medium using a specific ELISA. There were no statistically significant differences between the levels of GM-CSF secreted by WT and TTP-deficient BMSC in basal, unstimulated conditions, but the levels were barely detectable in this assay. On the other hand, after 24 hrs of stimulation with LPS, the medium from the WT cells contained  $2.5 \pm 0.6$  pg of GM-CSF/ml/ $\mu$ g of DNA (mean  $\pm$   
20 S.E.M. of 5 samples), versus  $14.6 \pm 4.5$  pg of GM-CSF/ml/ $\mu$ g of DNA in the medium from the TTP-deficient cells (5 samples). This 5.8-fold difference was statistically significant ( $p < 0.05$  using Student's t test). This result suggested that, as in the case of TNF $\alpha$  (11, 13), the absence of TTP resulted not only in an increase in the stability of GM-CSF mRNA, but also in the increased production of GM-CSF from these cells.

25

*Effect of TNF $\alpha$  receptor deficiency on GM-CSF mRNA stability in TTP-deficient mice.*

TNF $\alpha$  is well known as an inducer of GM-CSF synthesis (32-35); it has also been shown to increase the stability of GM-CSF mRNA (33). Therefore, there was a  
30 theoretical possibility that in the TTP-deficient cells, the increased stability of GM-CSF



mRNA could be secondary to the excess circulating TNF $\alpha$  levels that characterize these animals. To test this possibility, we generated mice that were deficient in TTP as well as in the two TNF $\alpha$  receptors (triple KO mice). In these mice, any phenotypic changes observed should be due to factor(s) other than TNF $\alpha$ .

5

To determine whether BMSC from the triple KO mice exhibited stabilized GM-CSF mRNA relative to their controls (animals lacking both TNF $\alpha$  receptors, but WT for TTP), LPS stimulation with and without actinomycin D was performed on their BMSC. In the absence of both TNF $\alpha$  receptors, GM-CSF mRNA levels in the BMSC  
10 changed in response to LPS in the same way as in the WT cells, appearing as two species of approximately 1.0 kb and 0.8 kb. However, in the triple KO cells, when TTP was also absent, a pattern identical to that observed in the TTP-deficient mice was seen, i.e., essentially the only form of GM-CSF mRNA present in these cells was the larger, polyadenylated species. In the double KO cells, the deadenylated form represented an  
15 average of  $41 \pm 6\%$  (n= 3) of the total GM-CSF mRNA versus  $7.5 \pm 0.4\%$  (n= 4) in the triple KO cells ( $p < 0.0005$ ). There was also marked accumulation of total hybridizeable mRNA in the triple KO cells compared to the double KO cells, with an approximately 4-fold increase observed at 3 hrs. Studies of GM-CSF mRNA stability after actinomycin D confirmed that the absence of TTP resulted in a prolonged half-life of  
20 GM-CSF mRNA, particularly the polyadenylated form, even in the absence of both TNF $\alpha$  receptor subtypes. In this experiment, the smaller, deadenylated form represented  $40 \pm 5\%$  (n= 5) of the total GM-CSF mRNA in the double KO cells versus  $6.1 \pm 0.9\%$  (n= 5) in the triple KO cells ( $p < 0.0001$ ). The calculated half-life of GM-CSF mRNA in the double KO cells was 63 min, but no decay was observed in the triple  
25 KO cells. These results establish that the absence of TTP *per se* is sufficient to inhibit deadenylation and increase the stability of GM-CSF mRNA, even in the absence of TNF $\alpha$  signaling.

The most important finding of the present study is that TTP appears to be a  
30 normal, physiological regulator of GM-CSF mRNA stability in the mouse. Using

BMSC derived from TTP-deficient mice, we showed that GM-CSF mRNA accumulation was markedly enhanced in cells lacking TTP relative to control cells after stimulation with either LPS or TNF $\alpha$ . Using the transcription inhibitor actinomycin D, it was demonstrated that this increased mRNA accumulation was due, at least in part, to an increase in GM-CSF mRNA stability in the TTP-deficient cells relative to control cells. These data and the data in Examples 2 and 3 indicate that cellular levels of both TNF $\alpha$  and GM-CSF mRNA are controlled to some extent by TTP. As in the case of TNF $\alpha$ , this leads to increased expression of GM-CSF protein from the TTP-deficient cells compared to control cells.

10

Concerning the mechanism of this effect, the TNF $\alpha$ , GM-CSF and interleukin-3 (IL-3) AREs are all so-called class II AREs, which contain several, usually tandem, AUUUA repeats. Co-transfection experiments in 293 cells, using artificial constructs in which the *c-fos* promoter was used to drive expression of the  $\beta$ -globin protein coding sequence linked to a 3'-ARE derived from either the TNF $\alpha$  or GM-CSF mRNA, revealed that TTP expression led to decreased accumulation of these "mRNAs", implicating the AREs in this process. In addition, when the half-life of *c-fos* mRNA, which contains a class I ARE, was estimated in the WT and TTP-deficient BMSC, there was no difference between the two genotypes, suggesting possible specificity of TTP for class II AREs. Direct binding studies demonstrated that TTP could bind directly to the TNF $\alpha$  ARE (see Examples 2 and 3). In additional co-transfection studies, TTP expression led to the destabilization of a somewhat truncated TNF $\alpha$  mRNA, in which the ARE was shortened from seven AUUUA repeats to 3.5, and the spacing between the ARE and a synthetic polyA tail of 33 residues was decreased to 0 b from the normal 300 b in the mouse (GenBank accession number X02611) (16). This TTP-induced mRNA destabilization was accompanied by the formation of a deadenylated intermediate. Thus, a primary role of TTP is to stimulate initially the process of deadenylation and ultimately the overall degradation of the mRNA.

30

Data from the present study firmly establish a physiological role for TTP as a promoter of GM-CSF mRNA deadenylation. In support of this conclusion are several types of data. First, in every experiment ( $n = 5$ ) in which BMSC from normal mice were used, Northern blotting of GM-CSF mRNA revealed that a substantial proportion (32-45 %) of the total GM-CSF mRNA was in a smaller form of approximately 0.8 kb, with the remainder in a larger form (approximately 1 kb). The existence of these two hybridizing forms of GM-CSF mRNA in mouse cells has long been noted in the literature (29, 37), and has been assumed to be due to use of alternate promoters or alternative transcription start sites. Using RNase H with oligo (dT), it was demonstrated that the smaller of the two species represented completely deadenylated GM-CSF mRNA. Thus, the larger of the two species is likely to contain the full polyA tail, calculated to be approximately 220 residues long in these cells. Although the two predominant species were routinely detected on Northern blots, between them on most blots there was a "smear" of intermediate-sized species, presumably representing partially deadenylated mRNA.

Second, in every experiment ( $n = 5$ ) performed with BMSC derived from TTP-deficient mice, there was marked accumulation of the larger, fully polyadenylated form of the GM-CSF mRNA relative to the smaller form, which represented only 6-16 % of the total GM-CSF mRNA. This is strong evidence that the normal control of GM-CSF deadenylation is regulated in some manner by TTP. Also, TTP binds and can be crosslinked to the normal, but not ARE-deleted, GM-CSF 3' UTR probes in cell-free studies. Co-transfection of plasmids expressing TTP with those expressing GM-CSF in human 293 cells also led to increased deadenylation of the mRNA and destruction of the mRNA body. These results demonstrate that TTP binds to the GM-CSF mRNA ARE and causes its deadenylation and destabilization.

Direct comparisons of translation rates of polyadenylated and deadenylated mRNAs have indicated that the polyA tail is necessary for normal rates of translation of some mRNAs (38-41). In the present studies, not only is total hybridizable GM-CSF

mRNA increased in cells from the TTP-deficient animals, but the 32-45 % of the total represented by the deadenylated mRNA species in the cells from the WT animals may not be normally translated.

- 5           These studies support a model for the severe inflammatory syndrome that characterizes the TTP-deficient mice, in which the absence of TTP leads to elevations in the steady state levels of mRNA for both  $\text{TNF}\alpha$  and GM-CSF, as well as the increased secretion of their encoded proteins. Since each cytokine is known to stimulate the secretion of the other, the initial hypersecretion of each could lead to an
- 10   interacting pathogenetic spiral in which the hypersecretion of each becomes greater with time.

- One of the most striking characteristics of the TTP-deficiency syndrome in mice was the exuberant myeloid hyperplasia noted in bone marrow and in extramedullary
- 15   sites. The present finding that TTP deficiency resulted in elevated levels of GM-CSF mRNA in BMSC, and increased secretion of GM-CSF from these cells, suggested that these and perhaps other cells might oversecrete this growth factor in the TTP KO mice, contributing in turn to the myeloid hyperplasia. In support of this possibility, mice deficient in the two types of  $\text{TNF}\alpha$  receptor as well as in TTP displayed myeloid
- 20   hyperplasia, both medullary and extramedullary. Therefore, the myeloid hyperplasia characteristic of the TTP-deficient mice may well be contributed to by chronic overstimulation by a growth-promoting factor such as GM-CSF.

#### References for Example 1

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## EXAMPLE 2

### Inhibition of Macrophage $\text{TNF}\alpha$ Production by TTP.

- Mice deficient in tristetraprolin (TTP), the prototype of a recently recognized
- 5 family of CCCH zinc finger proteins whose members have been identified in organisms ranging from man to yeast (5-8) were developed. Although the TTP-deficient mice appeared normal at birth, they soon developed a complex syndrome of inflammatory arthritis, dermatitis, cachexia, autoimmunity and myeloid hyperplasia. The TTP-deficiency syndrome could be reproduced in recombination activating gene 2 (RAG 2)
- 10 (-/-) immune-deficient mice by whole bone marrow transplantation from TTP-deficient mice after a lag period of several months, suggesting that transplanted macrophage progenitors might be among the cells responsible for the transplantability of the phenotype (10). Macrophages derived from fetal liver of TTP-deficient mice, or from bone marrow precursors or resident peritoneal macrophages from adult mice, exhibited
- 15 increased production of  $\text{TNF}\alpha$ , and increased levels of  $\text{TNF}\alpha$  mRNA, after stimulation with lipopolysaccharide (LPS) (10). In the best-studied example, bone marrow-derived macrophages from the knockout mice secreted approximately 5-fold more  $\text{TNF}\alpha$  than the control macrophages after incubation with LPS (1  $\mu\text{g/ml}$  for 4 hrs), while  $\text{TNF}\alpha$

mRNA levels were elevated about 2-fold in the knockout cells compared to controls (10).

To investigate the mechanism of this effect, we first evaluated the potential influence of TTP on TNF $\alpha$  gene transcription. We co-transfected a human TTP genomic construct, in which the instability-inducing 3'-untranslated region (UTR) of the TTP mRNA (8) was replaced by the 3'-UTR from the human growth hormone mRNA (11), with a TNF $\alpha$ -promoter/chloramphenicol acetyl-transferase (CAT)-reporter construct (Pro-CAT). This construct, which contained 2.3 kb of the mouse TNF $\alpha$  promoter linked to the CAT coding sequence and a 3'-UTR from a human growth hormone cDNA, was generously provided by Dr. Bruce Beutler, University of Texas/Southwestern Medical Center, Dallas, TX (12). Transfection of several cell types (chick embryo fibroblasts, NIH 3T3 mouse fibroblasts, and Rat-1 fibroblasts) showed that transfection with the TTP construct led to non-specific "squenching" of several co-transfected expression constructs, including Pro-CAT, SV2CAT (CAT driven by the SV40 promoter), and CAT driven by the *Xenopus* MARCKS gene promoter (13). In DNA dose-response studies in these cells, there was no evidence for preferential inhibition by the TTP constructs of Pro-CAT expression, when compared to the expression of the other co-transfected constructs. In human 293 cells, which express little if any endogenous TTP (11), transfection of TTP constructs did not significantly inhibit the expression of the same co-transfected constructs. When varying amounts of TTP DNA (0-10  $\mu$ g) were transfected into 293 cells along with Pro-CAT (5  $\mu$ g), average CAT expression (n=5 experiments) was completely unaffected by 1 and 5  $\mu$ g of TTP DNA, and was increased by 52% by 10  $\mu$ g of TTP DNA compared to 10  $\mu$ g of vector alone; none of these differences was statistically significant, using a paired t test with the Bonferroni correction for multiple comparisons (14). In parallel experiments (n=5) using TTP co-transfection with SV2CAT, TTP DNA at 1, 5 and 10  $\mu$ g caused statistically insignificant decreases in average CAT expression of 8%, 11% and 17%, respectively. Thus, there was no

evidence that TTP specifically inhibited Pro-CAT expression in this cell type, suggesting that the apparent effect of TTP to decrease macrophage TNF $\alpha$  mRNA and protein levels seen in previous studies (10) was not due to inhibited TNF $\alpha$  gene expression.

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We next evaluated the effect of TTP-deficiency on the stability of TNF $\alpha$  mRNA, which has a half-life reported to be 12 min in human monocytes (15) and 39 min in the murine macrophage cell line Raw 264.7 (16). In bone marrow-derived macrophages from wild-type (n=6) and TTP-deficient mice (n=6) that were stimulated with LPS (1  $\mu$ g/ml) (Sigma Chemical Co., St. Louis, MO) for 4 hr followed by treatment with actinomycin D (5  $\mu$ g/ml) (Sigma Chemical Co.), the half-life of TNF $\alpha$  mRNA in the macrophages lacking TTP was significantly increased (85 min) compared to the half-life of 39 min observed in the wild-type cells. The differences between the average TNF $\alpha$  mRNA levels were statistically significant at all times tested after 30 min of actinomycin D treatment, when compared by Student's t test. Northern analysis showed no evidence of stable mRNA degradation products in either the control or TTP-deficient macrophages. Similar studies with TTP (+/-) macrophages showed that TNF $\alpha$  mRNA decayed at the same rate as in wild-type cells, indicating that ~50% of normal cellular TTP concentrations (9) is sufficient to confer normal lability to TNF $\alpha$  mRNA.

These results suggest that TTP regulates TNF $\alpha$  mRNA levels at a post-transcriptional level. When TTP is present in the cells, TNF $\alpha$  mRNA is extremely labile, whereas its stability increases by more than two-fold in the absence of TTP. This increased half-life of TNF $\alpha$  mRNA in macrophages from the TTP-deficient mice is thus likely to be at least one of the factors responsible for the hypersecretion of TNF $\alpha$  by macrophages derived from these mice (10) and for the syndrome of TNF $\alpha$  excess that characterizes the TTP-deficient mice (9, 10).

Early studies on TTP cellular localization in fibroblasts demonstrated a nuclear localization in quiescent cells; the protein was found to either remain in the nucleus (7) or rapidly (< 5 min) translocate to the cytosol after mitogen stimulation (17). Our data, however, suggest a potential role for TTP in the cytosol, leading in some way to the destabilization of TNF $\alpha$  mRNA. This possibility was suggested by earlier studies in the murine macrophage cell line Raw 264.7, in which TTP appeared to be predominantly if not exclusively cytosolic (17). To determine the subcellular localization of TTP in normal macrophages under these conditions, cells treated with LPS or TNF $\alpha$  were labeled with <sup>35</sup>S-cysteine, separated into nuclear and cytosolic fractions, and then immunoprecipitated with anti-TTP antibody (17, 18). Both LPS and TNF $\alpha$  caused an increase in TTP labeling 4 hrs after stimulation that appeared to be exclusively cytosolic. Similar studies using Western blotting showed that increases in TTP protein were first detected in the cytosol 30 min after LPS stimulation, and persisted at high levels in the cytosol 2-4 hrs after stimulation. Nuclear TTP was not detectable in either the immunoprecipitation or the Western blot experiments.

These data suggested that TTP might participate in a novel negative feedback loop, in which cytosolic TTP is induced by the same stimuli that induce TNF $\alpha$  in macrophages, leading to instability of the TNF $\alpha$  mRNA and inhibition of TNF $\alpha$  secretion. To test this possibility, we first examined the expression of TTP mRNA in normal mouse bone marrow-derived macrophages stimulated with LPS (1  $\mu$ g/ml). After LPS treatment, TTP mRNA rapidly accumulated, peaking at 60 min. TNF $\alpha$  mRNA levels also increased, with a somewhat more prolonged time course. We next asked whether TTP was induced in primary macrophages stimulated with TNF $\alpha$ . Exposure of the cells to recombinant murine TNF $\alpha$  (10 ng/ml) (R & D Systems, Inc., Minneapolis, MN) resulted in marked increases in TTP mRNA levels, which peaked at 30-60 min; as shown in other studies (19), TNF $\alpha$  induced expression of its own mRNA, with levels peaking at 60-120 min.

Taken together, the results presented here suggest that TTP can regulate TNF $\alpha$  synthesis at a post-transcriptional level by promoting the turnover of TNF $\alpha$  mRNA. This represents a previously unknown negative feedback mechanism for regulating TNF $\alpha$  production. As the above data indicate and previous studies have shown (19),

5 TNF $\alpha$  promotes rather than inhibits its own synthesis and secretion. However, our data indicate that a negative feedback function can be performed instead by TNF $\alpha$ - or LPS-induced TTP. When TTP is absent, as in the TTP-deficient mice, the untrammelled self-stimulating property of TNF $\alpha$  almost certainly leads to the observed rapid downhill course in which many inflammatory processes are activated (9, 10).

10

TNF $\alpha$  mRNA contains several AU-rich elements (ARE) as well as considerable predicted secondary structure, both of which can influence mRNA stability (20).

To investigate whether the TNF $\alpha$  mRNA ARE was involved in the TTP effect,

15 we co-transfected 293 cells with constructs expressing TTP (21) and constructs (generously provided by Dr. Ann-Bin Shyu, University of Texas, Houston, TX) in which the AREs from TNF $\alpha$ , granulocyte-monocyte colony-stimulating factor (GM-CSF) and interleukin 3 (IL3) mRNAs were placed 3' of the *c-fos* promoter and the  $\beta$ -globin protein coding sequence (22). Co-expression of human TTP (21), either with a

20 genomic construct driven by the native human TTP promoter (5  $\mu$ g) or with a human TTP cDNA driven by the CMV promoter (5  $\mu$ g), markedly inhibited mRNA accumulation from all three constructs. Co-transfection with 0.01  $\mu$ g of the CMV-TTP construct or an unrelated CMV-MLP construct (23) had little or no effect. TTP mRNA was highly expressed in the cells transfected with 5  $\mu$ g of CMV-driven TTP, was

25 expressed to an intermediate extent after 5  $\mu$ g of the TTP genomic construct, and was barely detectable after the 0.01  $\mu$ g concentration of CMV-TTP. Parallel experiments in which a *c-fos* promoter/ chloramphenicol acetyl transferase (CAT) construct (24) was cotransfected with the TTP expression vectors revealed that the *c-fos* promoter was

completely unaffected by the expression of the genomic TTP construct or 0.01  $\mu$ g of the CMV construct, while it was inhibited by about 30% by 5  $\mu$ g of the CMV-TTP construct. Both *c-fos* promoter activity and  $\beta$ -globin mRNA accumulation were also unaffected by co-transfection of a plasmid containing CMV-driven MLP (5  $\mu$ g).

5

These results pointed to the ARE as the common element in these constructs responsible for the TTP stimulation of mRNA lability. To test whether TTP affected binding of cellular proteins to this region of the TNF $\alpha$  mRNA, we transfected 293 cells with a construct expressing epitope-tagged human TTP (21), and attempted to cross-link proteins in a cytosolic extract from these cells to a 153 bp probe from the mouse TNF $\alpha$  3'-UTR (25) that spanned the ARE. In untransfected cells, the radiolabeled mRNA probe was cross-linked to a major protein species of ~85 kDa. When extracts from TTP-expressing cells were used in a similar experiment, labeling of the 85 kDa protein decreased while a new binding protein of ~40 kDa appeared.

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Immunoprecipitation with an antibody specific to the epitope tag revealed that the 40 kDa protein was TTP itself. Expression of the tagged protein in these cytosolic extracts was confirmed by Western blotting; the protein that reacted with the epitope tag antibody also reacted with TTP antibodies. Essentially identical results were obtained when the probe was a 70 bp fragment consisting only of the TNF $\alpha$  ARE (25).

These data show for the first time that TTP binds directly to the ARE contained within the 3'-UTR of TNF $\alpha$  mRNA and probably other labile mRNAs, and that this binding somehow destabilizes these mRNAs. Many aspects of this interaction remain to be elucidated, including: The exact interaction sites on the mRNA and protein; the identities of the other proteins that are presumably recruited to this site to facilitate mRNA cleavage; qualitative or quantitative differences in TTP binding to other mRNAs with ARE-containing destabilizing elements; the effect of TTP serine

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phosphorylation (18) on mRNA binding; the potential overlapping effects of other members of the CCCH protein family; a possible role for TTP in regulating TNF $\alpha$  production from other cell types in which both proteins are expressed, such as keratinocytes and lymphocytes; the role of TTP in cells that do not synthesize appreciable TNF $\alpha$ ; its possible dysregulation by genetic or environmental factors or infectious processes in diseases of chronic TNF $\alpha$  excess; and many others. However, this demonstration of direct binding of TTP to TNF $\alpha$  mRNA, an interaction that presumably occurs in the cytosol, should permit the development of screening assays for compounds that potentiate, mimic or increase the specificity of this reaction, and may ultimately lead to novel drugs capable of inhibiting TNF $\alpha$  biosynthesis.

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21. Plamid H6E was first made by inserting a 3.7 kb ExoRI-XbaI fragment from the human genomic TTP clone (29) into the plasmid vector pBS+ (Stratagene). This insert contained ~1kb or promoter, the first exon, the intron, the second intron, and 30 bp of 3'-flanking region. For H6E.HGH3', a 597 bp NsiI-XbaI fragment in the 3'-UTR of the human TTP gene that contained five rapid degradation signal sequences was replaced by the entire 110 bp human growth hormone (HGH) 3'-UTR. The PCR primers used to amplify this fragment were (5'), 5'-GTGGCTTCTAGatgcatGGGTGGCATC-3', and (3'), 5-GAAGGACACtctagaGACAAAATGATGC-3', where the capital letters

represent the HGH sequences and the small letters represent the recognition sites for NsiI (5' primer) and XbaI (3' primer).

For CMV.TTP.tag, the influenza hemagglutinin (HA) epitope tag (31) was attached to the last amino acid of the human TTP cDNA (6) by the PCR-overlapping mutagenesis technique (30). The fusion insert containing the entire human TTP protein coding region and the HA epitope was then cloned into the HindIII site of the vector CMV.BGH3'/BS+. This vector was created by blunt-ligating a NruI-PvuII fragment from pRc/CMV2 (Invitrogen, San Diego, CA), which contains the hCMV promoter/enhancer and the bovine growth hormone polyadenylation signal, into the EcoRI and HindIII sites of pBS+ (Stratagene).

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25. RNA probes were prepared as follows: Plasmid p3'mTNF $\alpha$ , containing the mouse TNF $\alpha$  3'-UTR (bases 1110-1627 of GenBank accession number X02611) was created by RT-PCR, with the use of total cellular RNA from Raw 264.7 cells treated for 4 hrs with 1  $\mu$ g of LPS, as templates for RT. The 5' primer was 5'-CTTTCCgaattcACTGGAGCCTC-3', and the 3' primer was 5'-TAGAtctagaAGCGATCTTTATTTCTCTC-3', with the small letters indicating the restriction sites for EcoRI and XbaI, respectively. The resulting PCR fragment was digested and cloned into the EcoRI and XbaI sites of the vector pSK- (Stratagene). Plasmid pTNF $\alpha$  1197-1350 contained a 153 bp fragment that included the ARE of the mouse TNF $\alpha$  3'-UTR (1197-1350 of X02611); this was made using plasmid p3'mTNF $\alpha$  as the template. The 5' primer was 5'-

GATAagatctCAGGCCTTCC-3', and the 3' primer was 5'-GCCTtctagaTAAATACATTTCATAAGC-3'. The resulting PCR product was digested with BglII and XbaI (sites indicated by small letters in the primers) and cloned into the BamHI and XbaI sites of the vector pSK-. Plasmid pTNF $\alpha$  1281-1350 contained the seven AUUUA motifs of the TNF $\alpha$  ARE (1281-1350 of X02611). This was constructed using similar methods. Correct sequences of these plasmids were confirmed by dideoxy sequencing (Amersham Life Sciences Inc., Arlington Heights, IL). To radiolabel the RNA transcripts with  $\alpha$ -<sup>32</sup>P-UTP (800 Ci/mmol), plasmid TNF $\alpha$ 1197-1350 was linearized with XbaI and used as the template in the Promega Riboprobe in vitro Transcription System protocol (Promega, Madison, WI). The resulting product was precipitated with ammonium acetate and ethanol.

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27. Confluent dishes were washed three times with cysteine-free medium supplemented with 10% FCS. Cells were stimulated for 4 hrs in the same medium with control conditions (Con), 1  $\mu$ g/ml LPS or 10 ng/ml TNF $\alpha$ . For the last three hours of the incubation, 200  $\mu$ Ci/ml of <sup>35</sup>S-cysteine (NEN Life Sciences, Boston, MA) were added to the cultures. Cells were washed twice with ice-cold PBS, scraped into 10 ml of PBS and pelleted by centrifugation (1000 g for 5 min at 4°). Cells were then resuspended in 600  $\mu$ l of lysis buffer (50 mM Tris-HCl, pH 7.5; 50 mM NaCl; 3 mM MgCl<sub>2</sub>; 5% (v/v) glycerol; 0.5% (v/v) Nonidet P-40 (NP-40); 0.02% (w/v) sodium azide; 5 mM EDTA; 0.1 mM phenylmethylsulfonyl fluoride (PMSF); 20  $\mu$ g/ml soybean trypsin inhibitor; and 8  $\mu$ g/ml leupeptin), incubated on ice for 20 min, and lysed by passing 5 times through a 28 gauge needle attached to a 1 ml syringe with no dead space (Becton Dickinson and Company, Franklin Lakes, NJ). Nuclei (pellet after centrifugation at 1000 g for 5 min at 4°C) were washed once in ice-cold wash buffer (10 mM Tris-HCl, pH 7.5; 15 mM KCl; 1.5 mM MgCl<sub>2</sub>; 0.5 mM PMSF;

and 5% glycerol), centrifuged at 1000 g for 5 min at 4°C, and then resuspended and sonicated in the same volume of lysis buffer used initially to lyse the cells. The cytosolic fraction (supernatant) was clarified by centrifugation at 45,000 g for 30 min at 4°C, using a table-top ultracentrifuge (Beckman TL-100, rotor TLA.45, Beckman Instruments, Inc., Fullerton, CA). This method has been shown to result in clean cytosol-nuclear preparations, when assessed by Western-blotting with an anti-SP1 antibody. Cytosolic extracts matched by trichloroacetic acid-precipitable radioactivity and equivalent volumes of nuclear extracts were pre-cleared with pre-immune rabbit serum (1:100 dilution, 1 hr at 4°C) and protein A-sepharose (1 hr at 4°C), and then incubated overnight at 4°C in the presence of either pre-immune serum (1:100) or a 1:100 dilution of a polyclonal rabbit anti-mouse immune serum (17, 18). Immune complexes were recovered by centrifugation after the addition of protein A-sepharose, washed three times with wash buffer (50 mM Tris-HCl, pH 8.3; 150 mM NaCl; 1 mM EDTA; 0.5% NP-40), resuspended in 100 µl of SDS-sample buffer (28), and subjected to 9% SDS-polyacrylamide gel electrophoresis. Prior to autoradiography, gels were fixed and treated with Autofluor (National Diagnostics, Atlanta, GA).

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### EXAMPLE 3

#### **Evidence that TTP Binds to AU-Rich Elements and Promotes the Deadenylation and Destabilization of TNF $\alpha$ mRNA**

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In the present study, we asked whether the integrity of TTP's zinc fingers was necessary for its mRNA destabilizing and/or direct binding effect, and explored the nature of the cleavage of TNF $\alpha$  mRNA that resulted from TTP binding to its ARE in intact cells. Our data indicate that TTP exhibits zinc-finger-dependent ARE-binding activity, as well as a zinc finger-dependent ability to promote TNF $\alpha$  mRNA deadenylation and degradation. Through regulation of its cellular, subcellular and tissue-specific expression, induction kinetics and post-translational modification, this protein offers a myriad of potential mechanisms for regulating the stability of ARE-containing mRNAs.

15

#### **Materials and Methods**

##### **1. Plasmid construction.**

###### *a. Parent plasmids.*

The human TTP cDNA (43) and a human TTP genomic clone were obtained as described (23). Plasmid H6E was made by inserting a 3.7 kb EcoRI-XbaI fragment from the human genomic clone into the plasmid vector pBS+ (Stratagene, La Jolla, CA). This insert contained ~1 kb of promoter, the first exon, the single intron, the second exon, and 30 bp of 3' flanking region.

25

###### *b. Expression constructs.*

H6E.HGH3' was constructed as follows: a 597 bp NsiI-XbaI fragment in the 3' untranslated region (3' UTR) of H6E that contained five rapid degradation signal sequences was replaced by 110 bp of human growth hormone (HGH) sequence that encode the entire HGH 3' UTR (GenBank accession number M13438). The template

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used to amplify this fragment was pØGH (Nichols Institute Diagnostics, San Juan, CA). The PCR primers were, (5'), 5'GTGGCTTCTAGatgcatGGGTGGCATC<sup>3</sup>', and (3'), 5'GAAGGACACCTctagaGACAAAATGATGC<sup>3</sup>', where the capital letters correspond to the HGH sequences and the small letters correspond to the recognition sites for NsiI (5' primer) and XbaI (3' primer).

Construct CMV.hTTP.tag was made as follows: The epitope tag derived from the influenza hemagglutinin (HA) protein (21) was attached to the last amino acid of human TTP (hTTP) cDNA by the polymerase chain reaction primer-overlapping mutagenesis technique (24). The fusion insert that contained the entire human TTP protein coding region and the HA epitope (hTTP.tag) was then cloned into the HindIII site of the vector CMV.BGH3'/pBS+. The vector CMV.BGH3'/pBS+ was created by blunt-ligating a NruI-PvuII fragment from pRc/CMV2 (Invitrogen, Carlsbad, CA), which contains the hCMV promoter/enhancer and bovine growth hormone polyadenylation signal, into the EcoRI and HindIII sites of pBS+ (Stratagene). Expression of the fusion protein was confirmed by Western analysis of cytosolic extracts from human embryonic kidney (HEK) 293 cells transfected with the construct CMV.hTTP.tag, using the polyclonal antibody HA.11 (BAbCO, Richmond, CA) that recognized the tag. The zinc finger mutants C124R and C147R of CMV.hTTP.tag, which contained a single amino acid mutation at position 124 or 147, were made by the polymerase chain reaction primer-overlapping mutagenesis technique. In these mutants, the third cysteine in the CCCH motif of the first (C124) or the first cysteine (C147) in the second zinc finger were changed to arginine. Mutant S228A of CMV.hTTP.tag, in which the serine at position 228 (equivalent to the MAP kinase phosphorylation site S220 in mouse TTP (44)), was mutated to alanine using the same technique. All mutations were confirmed by dideoxy sequencing (Amersham/USB).

CMV.hTTP.EGFP was made as follows: Using the polymerase chain reaction primer-overlapping mutagenesis technique, an AgeI site was created immediately after the last amino acid of hTTP, so that the stop codon of hTTP was eliminated. When the

Asp718-AgeI fragment containing the entire hTTP coding region was inserted into the corresponding restriction sites of plasmid EGFP-N1 (Clontech, Palo Alto, CA), hTTP was fused to the N-terminus of EGFP in the same reading frame. The zinc finger mutants of CMV.hTTP.EGFP (C124R or C147R) were made by inserting BstEII-BamHI fragments of hTTP containing the mutations from the C124R or C147R mutants of CMV.hTTP.tag into the corresponding restriction sites in CMV.hTTP.EGFP. To make the construct H6E.EGFP, a promoterless fusion construct was created by first removing the CMV promoter from plasmid CMV.hTTP.EGFP by digestion with AseI and BglII, and then blunt-religating the remaining DNA. The hTTP-EGFP fusion plasmid without the promoter was then digested with EcoRI (a site in the multiple cloning site of the vector) and BstEII (a site in the hTTP coding region), and then an EcoRI-BstEII fragment from plasmid H6E containing ~1 kb of promoter, the first exon, the intron, and part of the second exon up to the BstEII site, was inserted into the corresponding sites in the fusion construct.

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CMV.mTNF $\alpha$  was made by first inserting a NarI-XbaI fragment containing bp 117-1325 of a mouse TNF $\alpha$  cDNA sequence (GenBank accession number X02611) into the HindIII (blunt-ligation) and XbaI sites of vector pSK- (Stratagene); an AseI-XhoI fragment containing the hCMV promoter/enhancer from pEGFP-N1 (Clontech) was then blunt-ligated into the XhoI site of the vector. Correct orientation of the promoter with respect to the mTNF $\alpha$  insert was confirmed by dideoxy sequencing. The mTNF $\alpha$  cDNA clone, provided by Dr. B. Beutler (The University of Texas Southwestern Medical Center, Dallas, TX), contained an incomplete 3' UTR that ended at bp 1325 (GenBank accession number X02611), with 33 adenylate residues attached to the last T.

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CMV.mTNF $\alpha$  (dARE) was made by deleting the ARE region (bp 1302-1325 of GenBank accession number X02611) of CMV.mTNF $\alpha$  using the polymerase chain reaction primer-overlapping mutagenesis technique. There were 28 adenylate residues attached to the last nucleotide (bp 1301 of GenBank accession number X02611) of this

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10049586-021202

construct.

2. Transfection of HEK 293 cells, Northern analysis, RNase H assay, and cytosolic extract preparation.

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HEK 293 cells were maintained in minimal essential medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Transient transfection of  $2 \times 10^6$  cells with CMV.mTNF $\alpha$  or other constructs in calcium-phosphate precipitates was performed as described previously (23, 24), except that the transfection mixture was allowed to stay on the cells for 16 to 20 hrs, and the glycerol shock step was omitted. In some experiments, pXGH5 (Nichols Institute Diagnostics) was also co-transfected to monitor transfection efficiency. Assays of released HGH were performed as described previously (23, 24).

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Twenty-four hrs after the removal of the transfection mixture, total cellular RNA was harvested from the HEK 293 cells using the RNeasy system (Qiagen, Valencia, CA). Northern blots were prepared as described (22). Blots were hybridized to a random-primed,  $\alpha$ - $^{32}$ P-labeled mouse TTP cDNA (22) or a  $\sim 1$  kb NarI-BglII fragment of mTNF $\alpha$  cDNA. Some blots were also hybridized to an  $\alpha$ - $^{32}$ P-labeled GAPDH cDNA probe (7) or a  $\sim 0.3$  kb fragment of mouse cyclophilin cDNA (bp 166-480, GenBank accession number X52803).

RNase H assays were performed by annealing RNA and oligonucleotide in 10  $\mu$ l of 50 mM KCl for 5 min at 50°C followed by an additional 10 min at 22°C. The mixture was incubated further at 37°C for 30 min in a buffer (4 mM HEPES-KOH (pH 8), 50 mM KCl, 2 mM MgCl $_2$ , 0.2 mM dithiothreitol (DTT) and 1  $\mu$ g/ $\mu$ l bovine serum albumin (BSA)) containing 0.8 unit of RNase H (Promega, Madison, WI), in a final volume of 25  $\mu$ l. The reaction mixture was then precipitated with sodium acetate and ethanol and the resulting RNA was subjected to Northern analysis.

30



Cytosolic extracts were prepared from HEK 293 cells 24 hr after the removal of the transfection mixture. The cells were incubated on ice for 20 min in a buffer consisting of 10 mM HEPES (pH 7.6), 3 mM MgCl<sub>2</sub>, 40 mM KCl, 5% (v/v) glycerol, 0.5% (v/v) Nonidet-P40, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 8 µg/ml leupeptin (lysis buffer). Lysis of the cells and maintenance of intact nuclei were carefully monitored by microscopy. The nuclei and cell membrane debris were removed by centrifugation at 16,000 g at 4°C for 15 min. Glycerol was added to the supernatant (cytosolic extract) to 20 % (v/v), and the resulting extract was stored at -70°C.

10

3. Analysis of RNA-protein complexes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretic mobility shift assay, and immunoprecipitation.

15

*a. Preparation of RNA probes*

Plasmid p3'mTNF $\alpha$  containing the mouse TNF $\alpha$  3'UTR (bp 1110-1627 of GenBank accession number X02611) was constructed by RT-PCR, using RNA from Raw 264.7 cells treated for 4 hrs with 1 µg/ml of LPS (Sigma, St. Louis, MO) as a template for RT. The 5' primer for PCR amplification was

20

5'CTTTCCgaattcACTGGAGCCTC3', and the 3' primer was 5'TAGAtctagaAGCGATCTTTATTTCTCTC3', where the small letters indicate the restriction sites for EcoRI and XbaI, respectively. The resulting PCR product was digested with these enzymes and cloned into the EcoRI and XbaI sites of the vector pSK- (Stratagene).

25

Plasmid pTNF $\alpha$  1197-1350, which contained a 153 bp fragment containing the AU-rich element (ARE) of mouse TNF $\alpha$  3'UTR (bp 1197-1350 of GenBank accession number X02611), was made by PCR using plasmid p3'mTNF $\alpha$  as a template, with a 5' primer, 5'GATAagatctCAGGCCTTCC3', and a 3' primer,

30

5'GCCTtctagaTAAATACATTCATAAGC3'. The resulting PCR product was digested

with BglII and XbaI (sites indicated by small letters in the primers) and cloned into the BamHI and XbaI sites of the vector pSK-.

Plasmid pTNF $\alpha$  1197-1300 (bp 1197-1300 of GenBank accession number X02611), containing only one AUUUA motif, was made using the TNF $\alpha$  3'UTR as template, with the M13-20 primer as the 5' primer, and a 3' primer, 5'CTGAtctagaAGTGCAAATATAAATAGAGG3'. The resulting PCR product was digested with EcoRV and XbaI (site indicated by small letters in the 3' primer) and cloned into the corresponding sites of the vector pSK-.

10

Plasmid pTNF $\alpha$  1281-1350 (bp 1281-1350 of GenBank accession number X02611) contained seven AUUUA motifs, five of them being overlapping UUAUUUAUU nanomers. This was constructed using the TNF $\alpha$  3'UTR as template, with a 5' primer, 5'GACTggatccTCTATTTATATTTGCAC3', and the M13 reverse primer as the 3' primer. The resulting PCR product was digested with BamHI (site indicated by small letters in the 5' primer) and XbaI and cloned into the corresponding sites of the vector pSK-.

15

Plasmid pTNF $\alpha$  1309-1332 (bp 1309-1332 of GenBank accession number X02611), containing four overlapping UUAUUUAUU nanomers, was constructed by inserting double-stranded oligonucleotides spanning bp 1309-1332 into the EcoRV-XbaI cloning sites of pSK-. Plasmid pTNF $\alpha$  1309-1332 (A/G), containing the same sequence except that the five As in the AUUUA motifs were replaced with Gs (see Fig. 1B), was made with the same technique.

25

Plasmid pTNF $\alpha$  1110-1325 (bp 1110-1325 of GenBank accession number X02611) was made by inserting the EcoRI-XbaI fragment of the mTNF $\alpha$  clone from Dr. Beutler into the corresponding sites of pSK-. This 248 base fragment contained five AUUUA motifs, three of them being clustered nanomers. There were 33 adenylate residues at its 3' end.

30

Correct sequences of all plasmid inserts were confirmed by dideoxy sequencing.

To label RNA transcripts with  $\alpha$ -<sup>32</sup>P-UTP (800 Ci/mmol), the above plasmids linearized with XbaI were used as templates, and the Promega Riboprobe *in vitro* Transcription Systems protocol was employed. The resulting product was precipitated  
5 with ammonium acetate and ethanol.

*b. Cross-linking of proteins to RNA*

Cytosolic extracts prepared from HEK 293 cells transfected with CMV.hTTP.tag or vector (20  $\mu$ g of protein) were incubated with 2 x 10<sup>6</sup> cpm of RNA  
10 probe in a 96-well plate at room temperature for 20 min in 20  $\mu$ l lysis buffer (without protease inhibitors). Heparin and yeast tRNA were added to final concentrations of 0.5  $\mu$ g/ $\mu$ l and 50 ng/ $\mu$ l, respectively, for an additional 10 min. The 96-well plate was then placed on ice and irradiated at 254 nm UV light in a Stratalinker (Stratagene) for 30 min at a distance of 5 cm from the light source. RNA not associated with protein was  
15 digested with 100 units of RNase T1 (Life Technologies, Inc) for 20 min at room temperature, and further digested with 1  $\mu$ g/ $\mu$ l of RNase A (Pharmacia Biotech, Piscataway, NJ) at 37°C for 15 min. The RNase resistant RNA/protein complexes were analyzed by SDS-PAGE (10% acrylamide gel) followed by autoradiography.

20 Identical samples were diluted to 0.5 ml in RIPA buffer and precleared with non-immune rabbit serum (1: 100 dilution, 1 hr at 4°C) and protein A-sepharose (Pharmacia Biotech) (1 hr at 4°C), and then incubated overnight at 4°C in the presence of either non-immune serum (1: 100) or a 1:100 dilution of a polyclonal antiserum. Immune complexes were recovered by centrifugation after the addition of protein A-  
25 sepharose, washed three times with wash buffer (50 mM Tris-HCl, pH 8.3; 150 mM NaCl; 1 mM EDTA; 0.5% (v/v) NP-40), resuspended in 100  $\mu$ l of SDS-sample buffer, and subjected to SDS-PAGE on 10% acrylamide gels and autoradiography.

*c. Western blotting.*

30 Cell extracts (5-50  $\mu$ g protein) were mixed with 1/5 volume of 5X SDS sample

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buffer (2), boiled for 5 min, then loaded onto 10% SDS-PAGE gels. Western blotting was performed by standard techniques. Membranes were incubated in Tris-buffered saline/0.5% Tween 20 (TBS/T) with either polyclonal antiserum HA.11 (1:2,500), or a rabbit antiserum to mouse TTP, 2640 (1:100; (38)), or a rabbit antiserum to human  
5 TTP, DU88 (1:100; (32)). Incubation of the membranes with second antibody and development were performed as described (6).

*d. RNA electrophoretic mobility shift assay.*

Cytosolic extracts prepared from HEK 293 cells transfected with either vector  
10 alone, or H6E.HGH3', or expression constructs driven by the CMV promoter (10 µg of protein), were incubated with  $1 \times 10^5$  cpm of RNA probe at room temperature for 20 min in 20 µl lysis buffer (without protease inhibitors). Heparin and yeast tRNA were added to final concentrations of 0.5 µg/µl and 50 ng/µl, respectively, for an additional 10 min. RNA not associated with protein was digested with 100 units of RNase T1  
15 (Life Technologies, Inc.) for 20 min at room temperature; the reaction mixture was then loaded onto a 6% non-denaturing acrylamide gel and subjected to electrophoresis at 250 V for 90 min, in 0.4 X Tris/borate/EDTA buffer.

4. Green fluorescent protein assays.

20

Cells were plated onto 100 mm dishes and transfected with hTTP-EGFP fusion constructs as described above. Twenty four hrs after the removal of the transfection mixture, the cells were transferred into 4-well Titertek slides (Fisher Scientific, Pittsburgh, PA) and incubated at 37°C overnight. The cells were washed once in PBS,  
25 fixed with 3.7% (v/v) formaldehyde for 5 min, and washed again with PBS. Glass cover slips were mounted using Vectashield fluorescent mounting media (Vector Laboratories, Burlingame, CA) and sealed with nail polish. Fluorescence microscopy was performed with a Zeiss confocal microscope model LSM 410 UV (Carl Zeiss, Inc., Thornwood, NY). Images were collected under 488 nm excitation using a 515-565 nm  
30 emission filter and a 100 x 1.4 NA oil immersion lens. Photographs were taken with a

16.1 sec scan.

## Results

### 5 Effect of TTP on TNF $\alpha$ mRNA species.

In most of the expression studies in 293 cells described below, we used a TNF $\alpha$  expression construct, CMV.mTNF $\alpha$ , that did not contain the entire native 3'UTR; instead, the TNF $\alpha$  sequence ended in the middle of the fourth AUUUA motif within the ARE (bp 1325 of GenBank accession number X02611; Fig. 1B) and was immediately  
10 followed by 33 adenylate residues encoded by the vector. To test whether this shortened ARE exhibited TTP binding activity, we compared TTP binding to a 3'-truncated RNA probe, comprising bases 1110-1325 of GenBank accession number X02611, to its binding to a non-truncated probe, comprising bases 1281-1350. This non-truncated probe contained the seven natural AUUUA motifs, five of them in  
15 clustered nanomers (Fig. 1B). We recently demonstrated that TTP could bind directly to a 1197-1350 probe (7). UV cross-linking of these probes to proteins in extracts from CMV.hTTP.tag-transfected cells indicated that TTP bound to the truncated probe 1110-1325 almost as well as to the probe containing all of the native AUUUA motifs (probe 1281-1350) (Fig. 1). A probe spanning bases 1197-1300, which only contained one  
20 AUUUA, exhibited barely detectable TTP binding activity under these conditions (Fig. 1).

We therefore used CMV.mTNF $\alpha$  in the cell expression studies described below, given the ability of TTP to bind to its mRNA ARE. The HEK 293 cells used in  
25 these studies normally do not express either TTP or TNF $\alpha$ , making these widely used cells a suitable intact cell system in which to study the interaction of TNF $\alpha$  mRNAs with transfected-expressed TTP. In addition, the expression of the truncated form of TNF $\alpha$  mRNA in these cells made possible for the first time the detection of a processing (probably deadenylated; see below) intermediate; this intermediate was not  
30 detectable when the native, full-length TNF $\alpha$  mRNA was expressed.

Both TTP and TNF $\alpha$  mRNAs were readily detected when the cells were transfected with either TTP or TNF $\alpha$  expression plasmids. There was a complex relationship found between the concentration of transfected CMV.hTTP.tag DNA and the resulting TNF $\alpha$  mRNA accumulation in the absence of actinomycin D treatment.

5 At low concentrations of transfected DNA (5 and 10 ng per plate), TNF $\alpha$  mRNA accumulation was ~ 20% of control, as determined by scanning densitometry of the Northern blot. This decrease in mRNA amount was accompanied by the appearance of a smaller species of mRNA, which first became apparent at 5-10 ng of DNA, but was more obvious at 50 ng. As described below, we believe this lower band to be the  
10 deadenylated form of the TNF $\alpha$  mRNA. Beginning at 50 ng DNA through all higher concentrations used essentially *all* TNF $\alpha$  mRNA was in this smaller form. However, the total *amount* of TNF $\alpha$  mRNA accumulation *increased* substantially at higher concentrations of DNA (see below) to reach a maximum of 214% of control at 500 ng. It remained high at 1  $\mu$ g before decreasing to 51% of control at 5  $\mu$ g. A similar but  
15 "right-shifted" dose-response relationship was present with the genomic TTP construct H6E.HGH3', which uses the weaker native TTP promoter rather than the CMV promoter; in this case, 2  $\mu$ g of DNA decreased total TNF $\alpha$  mRNA accumulation to 16% of control (n = 3); higher concentrations (5 and 10  $\mu$ g) resulted in continued expression of the smaller species in greater amounts.

20

The predominance of the smaller band and the almost complete absence of the larger band could be seen more readily after actinomycin D exposure, presumably because the larger band represented recently synthesized TNF $\alpha$  mRNA that was more likely to be full-length. In this case, 5 and 10 ng of CMV.hTTP.tag DNA resulted in  
25 less than 10% of control TNF $\alpha$  mRNA expression.

Because of the peculiar nature of this dose response, we performed four identical experiments with low concentrations of CMV.hTTP.tag, in which all samples were corrected for transfection efficiency using HGH expression, and were corrected  
30 for loading using Northern analysis of GAPDH mRNA and Phosphorimager analysis.

Compared to the vector alone control, there was a decrease in total hybridizing TNF $\alpha$  mRNA by 83% (to 17% of control) at 0.01  $\mu$ g CMV.hTTP.tag. This mean value increased to 173% of control at 0.05  $\mu$ g, and to 300% of control at 0.1  $\mu$ g DNA. Most of the hybridizing TNF $\alpha$  mRNA seen at the higher concentration of transfected

5 CMV.hTTP.tag was in the smaller form.

To determine whether transcription of CMV.TNF $\alpha$  was affected by the TTP expression plasmids, various amounts of either H6E.HGH3' or CMV.hTTP.tag were co-transfected into 293 cells with CMV.mTNF $\alpha$  or CMV.mTNF $\alpha$  (dARE). In the latter

10 construct, which was otherwise identical to CMV.mTNF $\alpha$ , 24 bp of the ARE were deleted (bp 1302-1325 of mTNF $\alpha$  cDNA; see Fig. 1B), resulting in a disrupted ARE that was incapable of binding TTP (see below). In this case, despite equivalent co-expression of TTP, the TNF $\alpha$  mRNA expressed from the CMV.mTNF $\alpha$  construct

15 containing the normal ARE was shortened in the normal way by the co-expressed TTP, while expression of the mutated CMV.mTNF $\alpha$  construct was unaffected either in apparent size or total accumulation by any concentration of co-transfected H6E.HGH3', and was minimally affected by CMV.hTTP.tag. Quantitation of these result by

PhosphorImager analysis and normalization for loading by cyclophilin mRNA showed that H6E.HGH3' at 5 and 10  $\mu$ g resulted in TNF $\alpha$  (dARE) expression that was 105%

20 and 98%, respectively, of the vector alone co-transfected control; whereas CMV.hTTP.tag at 0.01, 0.1 and 1  $\mu$ g resulted in TNF $\alpha$  (dARE) expression that was 110%, 97% and 73% of control, respectively. These experiments indicate that the effect of TTP to decrease TNF $\alpha$  mRNA accumulation at low concentrations of

CMV.hTTP.tag (i.e., 5 and 10 ng) was unlikely to be due to non-specific "squenching"

25 of transcription (7, 34), although this may have contributed to the modest decrease in TNF $\alpha$  mRNA expression seen with larger (5  $\mu$ g) amounts of CMV.hTTP.tag.

#### Evidence that TTP promoted deadenylation of TNF $\alpha$ mRNA.

The effect of TTP expression to cause shortening of the TNF $\alpha$  mRNA

30 suggested that TTP was promoting deadenylation of the TNF $\alpha$  mRNA poly A tail. To

evaluate this possibility, oligo dT<sub>12-18</sub> (P1) was added to total cellular RNA, and RNase H was used to remove the poly A tail (31). When this technique was used on RNA samples from cells co-transfected with CMV.mTNF $\alpha$  and either vector alone or TTP expression constructs (H6E.HGH3' in A, CMV.hTTP.tag in B and C), only the smaller of the two TNF $\alpha$  mRNA species remained. The smaller of the two mRNA species seen in the cells transfected with TTP constructs did not further decrease in size with the RNase treatment; this fact, and its identical size to the deadenylated TNF $\alpha$  mRNA from the control cells, indicated that the smaller form of the TNF $\alpha$  mRNA was deadenylated mRNA.

We also performed an RNase H experiment that used an oligonucleotide complementary to bp 506-528 of TNF $\alpha$  mRNA (GenBank accession number X02611; P2). The predicted sizes of the mRNA fragments from the resulting mRNA cleavage were ~400 b (5' portion) and ~810 b (3' portion). When RNA from 293 cells expressing both TNF $\alpha$  and TTP was analyzed after cleavage, most of the 3' TNF $\alpha$  mRNA fragment was in the form of the deadenylated smaller species, as compared to RNA harvested from cells expressing TNF $\alpha$  and vector alone. When both oligonucleotides were added together, the 3' fragment of the TNF $\alpha$  mRNA was of identical size in samples from control and TTP expressing cells. The size of the ~400 b 5' fragment was unaffected by TTP expression. These data confirmed that TTP promoted deadenylation of the TNF $\alpha$  mRNA.

Evidence for a precursor-product relationship between the upper and lower forms of TNF $\alpha$  mRNA.

In order to demonstrate that the larger, presumably polyadenylated form of TNF $\alpha$  mRNA could be converted to the smaller, deadenylated form by the presence of TTP, we analyzed the patterns of TNF $\alpha$  mRNA expression in cells co-transfected with small amounts of TTP expression constructs, before and after 4 hrs of exposure to actinomycin D (10  $\mu$ g/ml). As shown in the cells transfected with vector alone (BS+, 10  $\mu$ g; BS+, 5  $\mu$ g), there was *no* conversion of the larger form of TNF $\alpha$  mRNA to a



stable, smaller form in the absence of TTP, although the total amount of full-length mRNA decreased modestly after 4 hrs of actinomycin D. However, in the presence of TTP (10  $\mu$ g of H6E.HGH3' or 0.05- 0.5  $\mu$ g of CMV.hTTP.tag), actinomycin D exposure clearly led to the disappearance of the larger band, so that only the smaller band remained. Two additional experiments also examined intermediate time points. In both cases, the expression of TTP resulted in both forms of TNF $\alpha$  mRNA; the upper form then gradually disappeared after actinomycin D treatment.

Evidence that the ARE binding protein in 293 and macrophage extracts is TTP.

10 We next examined TNF $\alpha$  ARE binding activity in cytosolic extracts from bone marrow-derived macrophages from wild-type and TTP (-/-) mice that had been stimulated with LPS. After the cell extracts were UV cross-linked to the TNF $\alpha$  ARE probe and treated with RNases, an RNase-resistant RNA-protein complex was immunoprecipitated by an anti-TTP antibody but not by pre-immune serum. The  
15 macrophage TTP that was immunoprecipitated from the LPS-treated TTP (+/+) cells, but not from untreated (+/+) cells or from the treated or untreated TTP (-/-) cells, appeared as a smear with an average size of ~ 50 kDa, as compared to the apparent 40-44 kDa of mTTP expressed from CMV.mTTP in 293 cells. In our earlier studies, TTP migrated as a smear or multiple bands of ~ 35 to ~55 kDa (7, 44, 45). The difference in  
20 apparent molecular weights seen in the present experiment may have been due to differences in post-translational modification of the TTP protein, since, for example, its apparent molecular weight is known to increase after mitogen-stimulated phosphorylation (44). Despite these differences in apparent  $M_r$ , the identity of the immunoprecipitated protein as TTP cross-linked to  $^{32}$ P-labeled TNF $\alpha$  ARE was  
25 confirmed by the facts that the complex was precipitated from 293 cells that were transfected with TTP-expressing plasmids but not from cells transfected with vector alone; it was precipitated from 293 cells by three different antibodies including an antibody to the epitope tag (7); and that it was specifically immunoprecipitated from LPS-stimulated wild-type macrophages but not from unstimulated wild-type cells or  
30 from stimulated or unstimulated TTP-deficient cells.

These results indicate that the endogenous TTP formed after LPS treatment of normal macrophages can also bind to the TNF $\alpha$  ARE, and support the previously documented connection between the expression of TTP and the more rapid decay of TNF $\alpha$  mRNA in macrophages (7).

5

Involvement of the TTP zinc fingers in the binding of TTP to the ARE of TNF $\alpha$  mRNA.

We next evaluated the possible involvement of each of the two CCCH zinc fingers in the ARE-binding activity of TTP, using the TNF $\alpha$  probe 1197-1350 (see Fig. 10 1B). When cell extracts prepared from 293 cells that had been transfected with vector alone were used in UV cross-linking experiments, a major radioactive band of M<sub>r</sub> ~80,000 and several minor species were noted. When cell extracts prepared from 293 cells that had been transfected with hTTP expression constructs were used in UV cross-linking experiments, the extracts from cells transfected with either the wild-type 15 CMV.hTTP.tag or the S228A mutant (a point mutation at a MAP kinase phosphorylation site in the protein; 44) formed readily detectable RNase-resistant RNA-protein complexes of M<sub>r</sub> ~43,000 with the <sup>32</sup>P-labeled TNF $\alpha$  RNA probe, while simultaneously decreasing binding of the ARE probe to the endogenous cellular M<sub>r</sub> ~80,000 protein. However, extracts from cells transfected with 10  $\mu$ g of H6E.HGH3' 20 (human TTP driven by its native promoter and intron), with the C124R mutation in the CMV.hTTP.tag construct (the third C in the first zinc finger mutated to an R) or the C147R mutation in CMV.hTTP.tag (the first C in the second zinc finger mutated to an R) zinc finger mutants exhibited no detectable ARE binding activity. This indicates that single cysteine to arginine mutations in each of the TTP zinc fingers completely 25 prevented TTP binding to the TNF $\alpha$  ARE.

When the same UV cross-linked, RNase-treated extracts from cells transfected with CMV.hTTP.tag or H6E.HGH3' were immunoprecipitated with a polyclonal antibody to human TTP (DU88), or with a polyclonal antibody to mouse TTP (2640), 30 an RNA-protein complex of M<sub>r</sub> 40,000-50,000 was precipitated. This indicates that the

failure to see binding of TTP to the TNF $\alpha$  ARE probe in crude extracts from H6E.HGH3' transfected cells was simply due to much lower expression of the construct relative to the CMV construct. Neither antibody immunoprecipitated complexes from cells transfected with vector alone.

5

When the same UV cross-linked, RNase-treated extracts were immunoprecipitated with a polyclonal antibody to the epitope tag on TTP, the same RNA-protein complexes were precipitated from cells transfected with either the wild-type CMV.hTTP.tag or the S228A mutant, but only barely detectable complexes were  
10 seen in extracts from the cells transfected with either of the two zinc finger mutants in the CMV.hTTP.tag construct. The appearance of an immunoprecipitated complex of M<sub>r</sub> ~100,000 was clearly recognized by both antibodies to TTP and to the epitope tag, and most likely represented either TTP dimers or TTP complexed to a second protein of similar size as well as to the TNF $\alpha$  ARE probe.

15

To determine whether the mutant constructs used in these experiments expressed amounts of TTP protein that were equivalent to those expressed by the wild-type constructs, extracts prepared from 293 cells transfected with equivalent amounts of vector alone or either wild-type or mutant plasmids were subjected to Western blotting.  
20 Comparable amounts of fusion proteins were expressed from all four constructs, as recognized by the antibody to the epitope tag HA.11. An immunoreactive protein of M<sub>r</sub> ~100,000 was also seen by this technique, indicating that the integrity of the two zinc fingers in TTP is *not* required for the formation of these higher M<sub>r</sub> complexes, whether they are TTP dimers or TTP bound to another protein.

25

To further demonstrate that the binding of TTP to the TNF $\alpha$  ARE was specific, we made a mutant probe of pTNF $\alpha$  1309-1332 in which five of the flanking As in the AUUUA motif of the ARE sequence were mutated to Gs (see Fig. 1B). When this radiolabeled mutant probe was UV cross-linked to the extract from CMV.hTTP.tag  
30 transfected 293 cells, there was no detectable formation of the TTP complex, while the

amount of the  $M_r \sim 80,000$  complex was decreased but not eliminated. In contrast, the wild-type probe 1309-1332 could be readily cross-linked to TTP.

Electrophoretic mobility shift assays.

5           The specificity of TTP binding to the  $\text{TNF}\alpha$  ARE was also analyzed by electrophoretic mobility shift assays using  $\text{TNF}\alpha$  3' UTR probes. Incubation of probe 1197-1350 (containing the seven AUUUA motifs and some sequence 5' to them; see Fig. 1B) with a cytosolic extract prepared from 293 cells transfected with vector alone resulted in three major RNA-protein complexes, denoted I, II and III. When extracts  
10   from cells transfected with hTTP expression constructs were used, there were changes in the mobility of RNA-protein complexes I and II, while complex III disappeared. In a separate experiment, the extract from control cells was incubated with probe 1197-1350, and RNA-protein complexes were separated in a mobility shift assay. After the gel was exposed to UV light, complexes I, II, and III were eluted and analyzed by SDS-  
15   PAGE. Complexes I and II corresponded to an  $\sim 80$  kDa protein, and complex III corresponded to a  $\sim 55$  kDa protein. In the mobility-shift assays, the TTP-probe complex migrated approximately in the same positions as complexes I and II (as noted above in the UV cross-linking assays, the binding of TTP to the  $\text{TNF}\alpha$  mRNA ARE simultaneously decreased the binding of the ARE probe to the endogenous cellular  $M_r$   
20    $\sim 80,000$  protein). The same changes in protein-probe complex formation were seen when probes 1110-1325 (containing four AUUUA motifs; see Fig. 1B), 1281-1350 (containing seven AUUUA motifs), and 1309-1332 (containing only four clustered UUAUUUAUU nanomers) were used in the same assay.

25           In order to demonstrate that the binding of complexes I and II, and TTP, to the  $\text{TNF}\alpha$  ARE probes was specific, we also used a 54-nt region from the *c-fos* 3'UTR that has a 62% AU content without any AUUUA motifs (53) in the mobility shift assay. This 54-nt probe did not form complexes I and II with cytosolic extracts prepared from 293 cells transfected with vector alone, nor did it form a binding complex with extracts  
30   from TTP-expressing cells.

When one of the cysteine residues in either the first or the second zinc finger was mutated in construct CMV.hTTP.tag, extracts prepared from 293 cells transfected with these mutants no longer changed the mobility pattern of complexes formed when probe 1197-1350 was used. Similar results were obtained when probes 1110-1325 or  
5 1281-1350 were used.

To demonstrate that the mobility changes in complexes I and II were due to the binding of TTP to the TNF $\alpha$  RNA probe, an antibody to the epitope tag of the TTP fusion protein was added to the mobility shift assay. Although the antibody did not  
10 change the migration pattern of the RNA-protein complexes in extracts from control cells or from cells transfected with the two TTP zinc finger mutants, it retarded the migration of complexes formed in extracts from cells expressing either wild-type TTP or its S228A mutant. This supershift of the binding complex provided additional confirmation that the protein that bound to the RNA was TTP.

15

Importantly, the absence of TNF $\alpha$  ARE binding activity of the two TTP zinc finger mutants corresponded to their lack of effect on the conversion of TNF $\alpha$  mRNA to the smaller species in 293 cells. Normal amounts of the larger species of TNF $\alpha$  mRNA were present when CMV.mTNF $\alpha$  was co-transfected with either of the two TTP  
20 zinc finger mutant constructs, driven either by the CMV or the native human TTP promoter. The MAP kinase phosphorylation site mutant S228A, which retained its ability to bind to the TNF $\alpha$  ARE, also behaved like native TTP in promoting the shift to the smaller species of TNF $\alpha$  mRNA in intact 293 cells.

25 These experiments demonstrated the importance of the integrity of each of the zinc fingers in the binding of TTP to the TNF $\alpha$  ARE, as well as in the apparent deadenylation of the TNF $\alpha$  mRNA. These assays also indicated the importance of multiple cysteines in the zinc fingers, since mutating either the third C in the first finger or the first C in the second finger abolished TTP's RNA binding and cleavage-  
30 promoting activity.

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TTP is largely non-nuclear in these experiments

We previously demonstrated by differential centrifugation techniques that TTP was almost exclusively cytosolic in normal mouse macrophages (7) and in the macrophage cell line RAW 264.7 (45), although it had previously been localized to the nucleus of both quiescent (11, 45) and serum-stimulated (11) fibroblasts. For the present study, we constructed plasmids that expressed human TTP as a fusion protein with a modified green fluorescent protein (GFP), which normally is distributed throughout the cytoplasm and nucleus; this modified GFP localizes within the cell based on the peptides fused to it (12, 39). When 293 cells were transfected with EGFP-N1 (GFP alone driven by the CMV promoter), fluorescence was present in both the nucleus and cytoplasm. However, when the TTP-GFP fusion construct was transfected into 293 cells, the fluorescence was somewhat heterogeneous and appeared to be largely non-nuclear. This was true in cells transfected with both CMV.hTTP.EGFP, or the H6E.EGFP construct in which the hTTP-GFP fusion protein expression was driven by the native human TTP promoter and intron. Both the promoter and single intron of TTP play important roles in its expression (23, 24). Similar predominantly cytosolic distribution was seen in HeLa cells transfected with the same constructs.

To determine whether the hTTP-GFP fusion protein expressed in 293 cells was biologically active in these cells, we tested its ability to bind to the TNF $\alpha$  ARE probe in the cell-free assays and to promote the size-shift of TNF $\alpha$  mRNA in the intact cells. Both activities were exhibited by the hTTP-GFP fusion protein. We also demonstrated that a single C to R mutation in either the first or second zinc finger of human TTP markedly inhibited the ability of this human TTP-GFP fusion protein to cause the size shift in TNF $\alpha$  mRNA in 293 cells, or to bind to the TNF $\alpha$  ARE in cell-free extracts. These mutations did not appear to affect the pattern of distribution of the protein in the cells.

TTP can participate in the series of steps comprising the initial deadenylation followed by the ultimate degradation of at least some of those mRNAs containing so-

called type II AREs (8, 37), exemplified by  $\text{TNF}\alpha$ , GM-CSF and IL-3 (53). It seems likely that an early or possibly the first step in this interaction is the direct, zinc finger-mediated binding of TTP to the ARE, followed by a series of unknown steps that leads ultimately to removal of the polyA tail and subsequent (or simultaneous) mRNA degradation. That these events are likely to be physiologically significant is indicated by the results of our earlier studies with the TTP knockout mice and macrophages derived from them, in which the mice developed a  $\text{TNF}\alpha$  excess syndrome associated with increased macrophage production of  $\text{TNF}\alpha$ , due at least in part to increased stability of the  $\text{TNF}\alpha$  mRNA in the cells (6, 7, 46).

### **Figure Legend for Example 3.**

**Fig. 1A and 1B. UV cross-linking of human TTP to  $\text{TNF}\alpha$  mRNA ARE probes.**

**Fig. 1A:** Cytosolic extracts were prepared from 293 cells transfected with either 5  $\mu\text{g}$  of CMV.hTTP.tag or vector alone as described in Methods. Extract (20  $\mu\text{g}$  of protein) was incubated with the indicated  $^{32}\text{P}$ -labeled  $\text{TNF}\alpha$  RNA probes ( $2 \times 10^6$  cpm). The numbers at the top of each set refer to the base numbers in the mouse  $\text{TNF}\alpha$  mRNA, as shown in Fig. 1B. Probe 1110-1325 contained approximately 35% U residues; probe 1197-1300, 40%; and probe 1281-1350, 62%. Heparin and yeast tRNA were then added to decrease nonspecific binding. After UV cross-linking of the probes to cellular proteins, RNase T1 and A were added to digest probe not cross-linked to protein. The RNase-resistant RNA-protein complexes were resolved by 10% SDS-PAGE followed by autoradiography. Lanes 1: Probe alone (5,000 cpm). Lanes 2: Probe ( $2 \times 10^6$  cpm) treated with RNase T1 and A. Lanes 3: Extract (20  $\mu\text{g}$  of protein) from 293 cells transfected with vector alone (5  $\mu\text{g}$  of DNA). Lanes 4: Extract (20  $\mu\text{g}$  of protein) from 293 cells transfected with CMV.hTTP.tag (5  $\mu\text{g}$ ). The position of TTP cross-linked to  $^{32}\text{P}$ -labeled RNA is indicated by the arrow. The positions of protein molecular weight standards are indicated on the left.

**Fig. 1B:** Shown is a portion of the mTNF $\alpha$  mRNA 3'UTR (GenBank accession number X02611), from which the probes were derived. The five AU-rich nanomers are underlined. The five flanking As within the ARE that were mutated to form a non-binding probe are indicated in bold type.

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### References for Example 3

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#### EXAMPLE 4

##### **The tandem zinc finger domain from TTP and TTP-related proteins binds to AU-Rich elements and destabilizes mRNA**

5

#### **Methods**

##### **1. Plasmid construction.**

###### *a. Parent plasmids.*

The human (21) and mouse (2) TTP cDNAs were obtained as described. The  
 10 cDNAs encoding the Xenopus CCCH proteins XC3H-1, XC3H-3 and XC3H-4 were obtained as described (20).

###### *b. Expression constructs.*

Human TTP expression constructs H6E.HGH3' and CMV.hTTP.tag were made  
 15 as described (11). CMV.mTTP.tag, which contained the entire protein coding region of mouse TTP, was made using the same methods. CMV.hTTP(97-173).tag, CMV.hTTP(1-173).tag and CMV.hTTP(97-326).tag, which all contained the double zinc finger domain (aa 104-166 from ref. 21) and part of the protein sequence of human TTP, were made as described (11).

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CMV.http (97-173).tag contained essentially only the zinc fingers and seven flanking amino acids on both ends. CMV.hTTP(1-173).tag contained amino acids 1-173, and CMV.hTTP(97-326).tag contained amino acids 97-326 (the last amino acid) of human TTP.

5

CMV.CMG1.tag was made by inserting a PCR fragment containing the entire protein coding region of rat cMG1 (ref. 13, bp 108-1190 of GenBank accession number X52590), into the vector CMV.BGH3'/pBS+. The template cMG1 for the PCR reaction was generously provided by Dr. K.D. Brown (AFRC Institute of Physiology and  
10 Genetics Research, Babraham, Cambridge, UK). The epitope tag derived from the influenza virus hemagglutinin protein (22) was attached to the last amino acid of the cMG1 protein as described (23).

CMV.XC3H-1.tag and CMV.XC3H-3.tag were made by inserting PCR  
15 fragments containing the entire protein coding region of the corresponding Xenopus cDNA clones (20), as well as the epitope tag fused to the last amino acid of each protein, into the vector CMV.BGH3'/pBS+. CMV.U2AF35 was made by inserting a PCR fragment containing the entire protein coding region of the splicing factor U2AF35 (24) into the vector CMV.BGH3'/pBS+.

20

Plasmid pRSET B, which contained the entire coding region of U2AF35, was provided by Drs. B.R. Graveley and T. Maniatis (Harvard University, Cambridge, MA), and was used as a template in the PCR reaction.

25 CMV.mTNF containing a NarI-XbaI fragment spanning bp 127-1325 of a mouse TNF cDNA sequence (GenBank accession number X02611) was made as described (11). The mTNF cDNA clone, provided by Dr. B. Beutler (The University of Texas Southwestern Medical Center, Dallas, TX), contained an incomplete 3' UTR that ended at bp 1325 (GenBank accession number X02611), with 33 adenylate residues  
30 attached to the last T. This sequence is shown in Fig. 1 of reference (11).



2. Transfection of HEK 293 cells, Northern analysis, and cytosolic extract preparation.

HEK 293 cells were maintained in minimal essential medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transient transfection of  $1.5 \times 10^6$  cells with CMV.hTTP.tag, or with expression constructs containing the protein coding regions of the other CCCH zinc finger proteins, or with vector pBS+ alone in calcium-phosphate precipitates, was performed as described previously (23, 25), except that the transfection mixture was allowed to stay on the cells for 16 to 20 h, and the glycerol shock step was omitted. When cells were co-transfected with CMV.m TNF and CCCH protein expression constructs, human growth hormone expression plasmid pXGH5 (Nichols Institute Diagnostics, San Juan Capistrano, CA) was also co-transfected to monitor transfection efficiency.

Twenty-four h after the removal of the transfection mixture, samples were taken from the cell culture medium and human growth hormone released was assayed according to the manufacturer's protocol. Total cellular RNA was then harvested from the HEK 293 cells using the RNeasy system (Qiagen, Valencia, CA). Northern blots were prepared as described (2). Blots were hybridized to random-primed, -32P-labeled cDNA probes coding for various CCCH zinc finger proteins, including mouse TTP (2); Xenopus XC3H-1 and XC3H-3 (20); rat cMG1 (13); or splicing factor U2AF35 (24). Blots were also hybridized with a ~ 1 kb NarI-BglII fragment of a m TNF cDNA (11) and a ~ 0.3 kb fragment of mouse cyclophilin cDNA (bp 166 to 480; GenBank accession number X52803).

25

Cytosolic extracts were prepared from HEK 293 cells 24 h after the removal of the transfection mixture. The cells were incubated on ice for 20 min in a buffer consisting of 10 mM HEPES (pH 7.6), 3 mM MgCl<sub>2</sub>, 40 mM KCl, 5% (v/v) glycerol, 0.5% (v/v) Nonidet-P40, 2 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 8 µg/ml leupeptin (lysis buffer). Lysis of the cells and maintenance of intact nuclei

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were carefully monitored by microscopy. The nuclei and cell membrane debris were removed by centrifugation at 16,000 g at 4°C for 15 min. Glycerol was added to the supernatant (cytosolic extract) to 20 % (v/v), and the resulting extract was stored at -70°C.

5

3. Analysis of RNA-protein complexes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrophoretic mobility shift assay, and Western blotting.

*a. Preparation of RNA probes*

10 Plasmid pTNF 1281-1350 (bp 1281-1350 of GenBank accession number X02611) contained seven AUUUA motifs, five of them being overlapping UUAUUUAUU nonamers. This was constructed as described (11).

Plasmid pTNF 1309-1332 (bp 1309-1332 of GenBank accession number  
15 X02611), containing four overlapping UUAUUUAUU nonamers, was constructed by inserting double-stranded oligonucleotides spanning bp 1309-1332 into the EcoRV-XbaI cloning sites of pSK-.

Plasmid pTNF 1309-1332 (A/G) contained five Gs (underlined) replacing the  
20 five flanking As of bp 1309-1332 of GenBank accession number X02611 (UUGUUUGUUUGUUUGUUUGUUUUUU) and was constructed as described for pTNF 1309-1332.

Correct sequences of all plasmid inserts were confirmed by dRhodamine  
25 Terminator Cycle Sequencing (Perkin-Elmer, Foster City, CA).

To label RNA transcripts with <sup>32</sup>P-UTP (800 Ci/mmol), the above plasmids linearized with XbaI were used as templates, and the Promega Riboprobe in vitro Transcription Systems protocol was employed. The resulting products were precipitated  
30 with ammonium acetate and ethanol.

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*b. Cross-linking of proteins to RNA*

Cytosolic extracts prepared from HEK 293 cells transfected with CMV.hTTP.tag, or other zinc finger protein expression constructs, or vector (5 or 20 µg of protein) were incubated with  $1.5 \times 10^6$  cpm of RNA probe in a 96-well plate at room temperature for 20 min in 20 µl lysis buffer (without protease inhibitors). Heparin and yeast tRNA were added to final concentrations of 2.5 µg/µl and 50 ng/µl, respectively, for an additional 10 min. The 96-well plate was then placed on ice and irradiated at 254 nm UV light in a Stratalinker (Stratagene, La Jolla, CA) for 30 min at a distance of 5 cm from the light source. RNA not associated with protein was digested with 100 units of RNase T1 (Life Technologies, Inc) for 20 min at room temperature, and further digested with 25 µg of RNase A (Pharmacia Biotech, Piscataway, NJ) at 37°C for 15 min. The remaining RNA/protein complexes were analyzed by SDS-PAGE (12% or 16% acrylamide gel) followed by autoradiography.

*c. Western blotting.*

Cell extracts (5-50 µg protein) were mixed with 1/5 volume of 5X SDS sample buffer (26), boiled for 5 min, then loaded onto 12% or 16% SDS-PAGE gels. Western blotting was performed by standard techniques. Membranes were incubated in Tris-buffered saline/0.3% Tween 20 (TBS/T) with either polyclonal antiserum HA.11 (1:2,500) or an antiserum to U2AF35 (27). Incubation of the membranes with second antibody and development were performed as described (8). For some blots, 125I-protein A (0.2 µCi/ml in TBS/T; Amersham, Arlington Heights, IL) was used in place of second antibody.

*d. RNA electrophoretic mobility shift assay.*

Cytosolic extracts prepared from HEK 293 cells transfected with either vector alone or expression constructs driven by the CMV promoter (5 or 20 µg of protein) were incubated with  $2 \times 10^5$  cpm of RNA probe at room temperature for 20 min in 20 µl lysis buffer (without protease inhibitors). Heparin and yeast tRNA were added to final concentrations of 2.5 µg/µl and 50 ng/µl, respectively, for an additional 10 min.

RNA not associated with protein was digested with 100 units of RNase T1 (Life Technologies, Inc.) for 20 min at room temperature; the reaction mixture was then loaded onto a 6% non-denaturing acrylamide gel and subjected to electrophoresis at 250 V for 90 min, in 0.4 X Tris/borate/EDTA buffer.

5

#### 4. Expression of XC3H-4 protein and its fragments in *Xenopus* oocytes.

##### *a. Preparation of XC3H-4 RNA in vitro*

cDNAs encoding various regions of the XC3H-4 protein (20), full length (aa 1-276); from amino acids 1 to 120 (containing the tandem CCCH zinc fingers of the TTP-  
10 type); and from amino acids 121 to 276 (containing the second pair of CCCH zinc fingers) were inserted into the BglII cloning site of plasmid pSP64TEN (a gift from Dr. Douglas Melton, Harvard University, Cambridge, MA). The epitope tag derived from the influenza hemagglutinin protein (22) was attached to the last amino acid of each of the peptides as described (23). Correct sequence of the inserts was confirmed by  
15 dRhodamine Terminator Cycle Sequencing (Perkin-Elmer). The plasmids were linearized by XbaI digestion, and were used as templates to synthesize RNA in vitro. The RNAs were prepared with the use of the mMESSAGEMACHINE SP6 Kit (Ambion, Inc., Austin, TX) following the manufacturer's protocol.

##### 20 *b. Microinjection of oocytes*

Ovary was removed from adult *Xenopus* females (*Xenopus* I, Ann Arbor, MI), and stage VI oocytes were separated from the ovary and manually defolliculated.

Oocytes were allowed to recover for 16 h at 18°C in buffer OR-2 (5 mM HEPES (pH 7.8), 82.5 mM NaCl, 2.5 mM KCL, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 3.8 mM NaOH).

25 Oocytes were injected with 30- 50 ng of mRNA and incubated for 24 h at 18°C. Oocyte cytosolic extracts were prepared as described above for 293 cell extracts.

## Results

The other two human members of the mammalian CCCH double zinc finger  
30 protein family, ERF1 (cMG1, TIS11b) and ERF2 (TIS11d), share strikingly similar

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amino acid sequences in the tandem zinc finger (TZF) region with TTP (Fig. 2, underlined). Although the carboxyl termini exhibit some sequence similarities, there are major differences between TTP and the other two proteins, while the amino acid sequences of ERF1 and ERF2 are more closely related to each other (Fig. 2).

5

To determine whether these other two family members shared TTP's ability to bind to the ARE region of the TNF mRNA and destabilize it, ARE binding studies were performed using proteins expressed in HEK 293 cells, and co-transfection assays with the TNF mRNA expression construct were performed in the same cell type.

10 Besides the TTP expression constructs, the new expression constructs used were made from rat cMG1 (13), which is the rat homologue of mouse TIS11b, human ERF1, and *Xenopus* XC3H-2; and *Xenopus* XC3H-3 (20), which is the *Xenopus* homologue of mouse TIS11d and human ERF2.

15 *Effects of TTP-related proteins on TNF mRNA*

In the expression studies described below, we used a previously described (11) TNF expression construct, CMV.mTNF. This construct does not contain the full 3'UTR of mTNF; instead, it ends at base 1325 (of GenBank accession number X02611) followed by 33 adenylate residues encoded by the cDNA. The expression of TNF  
20 mRNA from this construct allowed the detection of both the adenylated and deadenylated forms of this mRNA in the presence of TTP (11). TTP has similar effects on the full-length mouse TNF mRNA (data not shown), but for technical reasons involving size overlap with the 18S ribosomal RNA, these are more difficult to quantitate.

25

The HEK 293 cells used in the transfection experiments do not express endogenous TNF or TTP mRNA (11). Quantitation of Northern blot mRNA expression was determined by Phosphorimager, and was corrected for transfection efficiency by HGH secretion and for gel loading by quantitating endogenous cyclophilin mRNA  
30 levels. When these cells were co-transfected with CMV.mTNF and a range of

concentrations of the human TTP expression construct CMV.hTTP.tag, the mTNF mRNA exhibited a characteristic expression pattern. At a low concentration of TTP DNA (0.005  $\mu$ g per dish of cells), the total amount of m TNF mRNA was reduced to ~40% of control in this experiment. When the amount of co-transfected TTP DNA was increased to 0.01  $\mu$ g of DNA, the total amount of hybridizeable TNF mRNA was further reduced to 23% of control. When 0.1 and 1  $\mu$ g of TTP were co-transfected, a smaller species of m TNF mRNA was increased in intensity, while the upper band decreased markedly. We have previously shown by RNase H experiments that the lower band is a deadenylated species of TNF mRNA (11). At these higher concentrations of TTP DNA, the total accumulated hybridizeable mTNF mRNA was actually greater than that seen in the cells co-transfected with vector alone.

After correcting for transfection efficiency, the amounts of hybridizeable mTNF mRNA from cells co-transfected with 0.1 or 1  $\mu$ g of CMV.hTTP.tag were 254% and 481% of control, respectively. The apparent decrease in hybridizable mTNF mRNA seen at the highest concentration of TTP DNA (1  $\mu$ g) is likely to be due to the global inhibition of transcription seen at this level of TTP expression, as noted previously (11); this was reflected in a marked decrease in HGH expression from these cells. These results are similar to those described previously (11), in which Phosphorimager values from four independent experiments were normalized for both transfection efficiency and gel loading and then averaged. In that study, an average decrease of TNF mRNA to 17% of control was seen at 10 ng of CMV.hTTP.tag DNA; this value increased to 173% and 300% of control at 50 and 100 ng of DNA, respectively. The mechanism of the increased accumulation of the deadenylated species of TNF mRNA seen at higher TTP expression plasmid concentrations is not known, but is a consistent and highly reproducible finding (11).

In the same co-transfection experiment, we tested the ability of the two TTP-related proteins to destabilize TNF mRNA and to promote the formation of the deadenylated species. When either CMV.CMG1.tag (a rat cMG1 expression plasmid,

representing the cMG1/TIS11b/ERF1 proteins) or CMV.XC3H-3.tag (a *Xenopus* XC3H-3 expression construct, representing the TIS11d/ERF2 proteins) was co-transfected with CMV.mTNF into 293 cells, each exhibited a similar pattern to TTP in influencing the accumulation of TNF mRNA. With very low amounts of co-transfected

5 CMV.CMG1.tag DNA, 0.005 and 0.01  $\mu$ g, the total hybridizeable amounts of TNF mRNA were decreased to 40% and 27% of control, respectively. The accumulation of the smaller species of TNF mRNA was obvious at 0.1 and 1  $\mu$ g of co-transfected CMV.CMG1.tag DNA; total hybridizeable TNF mRNA was 106 % and 663% of control, respectively. Expression of the *Xenopus* protein, XC3H-3, resulted in a similar

10 pattern. Transfection of low concentrations of CMV.XC3H-3.tag DNA (0.005 and 0.01  $\mu$ g) caused a decrease in total TNF mRNA to 57% and 49% of control, respectively. At 0.1  $\mu$ g of CMV.XC3H-3.tag DNA co-transfection, the characteristic two sizes of TNF mRNA were detected, while at 1  $\mu$ g of CMV.XC3H-3.tag DNA, the deadenylated species of the mRNA accumulated to 461% of control.

15

As a control, we used the human RNA splicing factor U2AF35 (24). This protein contains two putative zinc fingers of the CCCH class, which, instead of being 18 amino acids apart, are widely separated by 116 amino acids, and are also not preceded by the YKTEL lead-in sequence. The U2AF35 protein is the smaller subunit

20 of the essential splicing factor U2AF (24). Its heterodimeric complex with U2AF65 is thought to be required for recognition of the 3' splice acceptor site in pre-mRNA splicing (for reviews see (28,29)). U2AF35 has also been shown to interact with other proteins involved in splicing, such as SC35, SF2/ASF, tra and tra2, and was originally not thought to directly bind to RNA (27, 30); however, this conclusion has been revised

25 recently (31-33). Although endogenous U2AF35 mRNA in 293 cells was readily detectable, the transfection markedly increased the expression of this mRNA and protein. In this co-transfection experiment, the TNF mRNA was not affected by the expression of increasing amounts of U2AF35. This is in contrast to the effects of two other TTPs, mouse (2) and *Xenopus* (XC3H-1 (20)), both of which behaved like their

30 human counterpart in this assay.

*Binding of CCCH zinc finger proteins to the ARE of TNF mRNA*

Using the 70 b <sup>32</sup>P-labeled TNF ARE probe (bases 1281-1350 of Genbank accession number X02611), we performed UV cross-linking experiments with extracts from 293 cells that had been transfected with constructs expressing these CCCH zinc finger proteins driven by the CMV promoter. In addition to human TTP, both rat cMG1 and Xenopus XC3H-3) were crosslinked by the TNF ARE probe. Two major crosslinked proteins bands were formed with XC3H-3; one at Mr ~ 48,000, presumably representing the intact protein, and one at Mr~ 32,000, presumably representing a proteolytic fragment. Human U2AF35 protein, whose widely spaced zinc fingers lack the YKTEL lead-in sequence, did not form detectable complexes with the TNF ARE probe. The identities of the endogenous 293 cell proteins that cross-linked to this probe, of Mr ~ 85,000, ~70,000, ~46,000 and ~35,000 are unknown; their possible relationship to other ARE binding proteins described in the literature has been discussed previously (11).

15

The ARE binding activity of the two TTP-related proteins was also tested in RNA mobility shift assays. As we have shown (11), the TNF ARE probe formed three RNA-protein complexes (I, II and III) with a control extract prepared from 293 cells transfected with vector alone. When an extract from cells transfected with CMV.hTTP.tag was used, new complexes were formed while complexes I, II and III seen in the extract from vector-transfected cells decreased or disappeared.

20

Formation of new RNA-protein complexes was likewise observed when extracts from 293 cells transfected with either CMV.CMG1.tag or CMV.XC3H-3.tag were used in the assay. Considerable radioactivity also remained in the gel wells when extracts containing these two proteins were used. Human U2AF35 RNA splicing factor did not form detectable complexes with the TNF ARE probe under these conditions. Expression of these proteins from the expression constructs was readily detectable using antibodies to the epitope-tagged proteins.

30



*Binding of TTP and related proteins to mutant TNF ARE probes*

We previously determined that the flanking A nucleotides of the AUUUA motifs in the TNF ARE were essential for binding of TTP to the TNF ARE (11). We next evaluated the ability of the TTP-related proteins to bind to mutant ARE probes.

5 We first compared a longer TNF ARE probe (representing b 1281 to 1350 of GenBank accession number X02611) that contained seven AUUUA motifs to a probe (b 1309-1332) that contained only four of the AUUUA sequences (Fig. 3B). Using probe 1281-1350, cytosolic complexes of Mr~85,000, ~ 70,000, ~ 46,000 and ~ 35,000 were seen when vector-transfected cell extracts were used; these were less apparent when the  
10 shorter probe 1309-1332 was used (Fig. 3A, compare lane 2 with 7, or lane 6 with 11). Extracts prepared from 293 cells transfected with the hTTP, cMG1, and XC3H-3 expression constructs all formed complexes with both long and short probes (Fig. 3A, lanes 3-5, 8-10).

15 We next studied the binding specificity of TTP and its related proteins to a mutant of the short probe 1309-1332. None of the proteins was able to form detectable complexes with probe 1309-1332 (A/G) (Fig. 3B, mutation sites indicated by triangles), a mutated ARE probe in which the flanking A residues in the AUUUA motif were substituted with Gs (Fig. 3B, lanes 13-17).

20

*Characteristics of a fourth CCCH protein.*

We previously identified a fourth prospective family member of the CCCH TZF protein family in *Xenopus* (accession number AAD24210 (20)). This protein, XC3H-4, contained two zinc fingers spaced 18 amino acids apart that contained all of the  
25 hallmarks of the TZF domains from the three proteins discussed above; in addition, it contained two additional, more carboxyl-terminal CCCH zinc fingers, spaced more closely together and containing more degenerate lead-in sequences (20). Database searches revealed sequence similarity to the amino terminal portions of two CCCH proteins from zebrafish (34) and carp (35). Subsequent correction of the fish DNA  
30 sequences (CAA71245.2 for carp CTH1, CAB55775.1 for zebrafish CTH1) showed

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apparent homology with the *Xenopus* sequence over the entire lengths of the proteins (Fig. 4). To our knowledge, mammalian homologues of these proteins have not been cloned to date.

5        Attempts to express the *Xenopus* member of this group, XC3H-4, or various subdomains, in 293 cells failed to yield significant levels of mRNA or protein. However, significant expression of protein was readily achieved by injecting mRNA into *Xenopus* oocytes; extracts from these oocytes were then used in ARE crosslinking studies. Using the <sup>32</sup>P-labeled TNF ARE probe (bases 1281-1350 of GenBank  
10    accession number X02611), we performed UV cross-linking experiments with extracts from *Xenopus* oocytes that had been injected with in vitro transcribed RNAs encoding the full length, the first half (aa 1 to 120 of accession number AAD24210), or the second half (aa 121 to 276) of the XC3H-4 protein. Similar to human, mouse, or *Xenopus* (XC3H-1) TTP expressed in 293 cells, extracts prepared from oocytes  
15    injected with *Xenopus* XC3H-4 RNA that encoded the full-length protein were crosslinked by the TNF ARE probe, while no probe-protein complex was detectable when extracts of buffer-injected oocytes were used. When an extract from oocytes injected with RNA encoding the first 120 amino acids of XC3H-4 protein was used in the UV cross-linking assay, a probe-protein complex with an apparent Mr 15,000 was  
20    observed. This complex formation is presumably due to the TZF domain that is related to the one in TTP (see Fig. 5B). When an extract from oocytes injected with RNA encoding the second half of the XC3H-4 protein (aa 121-276) was used in the UV cross-linking assay, no probe-protein complex could be detected. The second half of the protein contains a pair of CCCH zinc fingers that each have the internal spacing of the  
25    TTP-type zinc fingers, but the two fingers are separated by only 7 amino acids and they lack the R(K)YKTEL lead-in sequence. Human U2AF35 protein also did not form detectable complexes with the TNF ARE probe. Expression of the XC3H-4 protein and its fragments from the RNA-injected oocytes was readily detectable using antibodies to the epitope-tagged proteins.

*Interaction of TTP fragments with TNF mRNA*

As shown in Fig. 2, the human proteins ERF1 and ERF2 exhibit much greater similarity in amino acid sequence with TTP within the TZF domain than in other regions of the proteins, suggesting that the common TZF domain may be the key

5 component of these proteins that binds to and regulates the stability of ARE-containing mRNAs. We have already shown that the integrity of both zinc fingers is necessary for TTP binding to the TNF ARE in cell-free assays, and for destabilizing TNF mRNA in cell transfection experiments (11).

10 We asked next whether the TZF domain alone was sufficient for TTP to interact with the TNF mRNA. Three expression constructs were prepared that all contained the TZF domain; this spans amino acids 104-166 of human TTP (21) GenBank accession number M63625). CMV.hTTP(1-173).tag contained amino acids 1-173; CMV.hTTP(97-326).tag contained amino acids 97-326 (the carboxyl terminus of the  
15 protein); and CMV.hTTP(97-173).tag contained the double zinc finger domain flanked by seven amino acids at each end.

When cell extracts from 293 cells expressing these protein fragments were used in UV cross-linking experiments using TNF ARE probes, TTP and all of its fragments  
20 could be cross-linked to a longer probe containing the full ARE (b 1281-1350) as well as a shorter probe containing only four AUUUA motifs (b 1309-1332). Neither the full-length TTP protein, nor any of its fragments, was able to form a detectable complex with the mutant probe 1309-1332 (A/G), in which the flanking As of its four AUUUA motifs were replaced by Gs.

25

In RNA mobility shift assays using the TNF ARE 1309-1332 probe and 293 cell extracts prepared from cells transfected with these TTP expression constructs, each of these TTP fragments, like the full-length protein, was able to form a probe-protein complex. When an epitope-tag antibody was included in the mobility shift assay, all of

these TTP fragments formed super-shifted complexes, while there was no super-shifted complex formation with extracts from vector-transfected 293 cells.

Although the amount of mRNA and protein expression from these TTP  
5 fragment expression constructs was somewhat decreased relative to full-length TTP, readily detectable amounts of these fragments were seen by probing a Western blot with an antibody to the epitope tag.

We then tested the ability of these truncated forms of TTP to cause  
10 deadenylation and/or degradation of the TNF mRNA in intact cells. When 293 cells were co-transfected with CMV.m TNF and either CMV.hTTP(1-173).tag or CMV.hTTP(97-326).tag, the TNF mRNA exhibited the shortening to the deadenylated form seen with full-length TTP. When construct CMV.hTTP(97-173).tag was used, the  
15 characteristic two bands of TNF mRNA were seen at both 1 and 5 µg of transfected DNA. These data indicated that the 77 amino acid peptide containing the TZF domain alone was capable of promoting the decrease in size of the TNF mRNA, which we have attributed to its deadenylation (11). Concentration-response experiments showed that the apparent differences in potency of these constructs to promote deadenylation of the  
20 TNF mRNA appear to be due to differences in expression.

#### *Alignment of TZF domains of known CCCH proteins*

In order to identify critical sequence requirements for ARE binding, we have begun an analysis of the TZF domain of the vertebrate CCCH proteins. We aligned the TZF domains from the four vertebrate proteins discussed here, making the assumption  
25 (borne out in every case in which it has been tested directly) that the domains from homologues from other animal species would bind to the ARE probe similarly to the prototype protein. We identified TZF domains from larger amino acid sequences already in GenBank (with one exception), as listed in the legend to Fig. 5B. The single exception is from an unpublished *Xenopus* EST, in which a single open reading frame  
30 predicted a *Xenopus* allelic variant of XC3H-33. This is now listed as XC3H-3.2, with

the original allele (20) listed as XC3H-3.1. The 64 amino acid TZF domains from all of these proteins were aligned using the program Pileup from GCG. In Fig. 5A is illustrated the dendrogram produced by these alignments; despite the facts that these proteins are much more disparate outside of the TZF domains than within them, the alignment program still aligned them into homologous groups (Fig. 5A). The alignment itself is pictured in Fig. 5B. To simplify the discussion of the alignment, the amino acids within the TZF domains are numbered from 1-64.

Examination of Fig. 5B reveals that 34 of the 64 amino acids in the TZF domains (53%) have been conserved among all four proteins from species as diverse as human, *Xenopus* and zebrafish. These include the RYKTEL lead-in sequence for the first zinc finger, the lead-in sequence KYKTEL in the second zinc finger, and several other amino acids in the inter-finger 18 amino acid spacer, including a G residue at position 27, an acidic residue at 30, an L at 31, an H at 37, and a P at 38. Within both zinc fingers, the canonical CCCH residues were conserved. Within the first finger, an E residue was conserved at position 12, a G at 14, a Y at 18, a basic residue at 21, and a QFA at 23-25. Within the second finger, a G residue was conserved at position 52, a Y at 56, an R at 59, an F at 62, and a branched chain amino acid at 63.

The TZF domains also appeared to contain protein-specific "signatures", which allowed the domains from a given protein (e.g., TTP) to be grouped appropriately with its homologues (see Fig. 5A), despite the great evolutionary distance between the animal species examined. For example, TTPs from human, cow, rat, mouse, and *Xenopus* all contain T residues at position 9, S residues at position 11, A residues at position 20, N residues at position 35, HK at 46 and 47, YL at 49 and 50, and S residues at position 58, differentiating the TTP homologues from all other proteins examined. Similarly, the cMG1 homologues all contain D residues at position 20 and I residues at position 28, distinguishing them from the others. The TIS11D proteins had as their only signature an F residue at position 28. The more distantly related XC3H-4

proteins also contained signature amino acids, including SR at 8 and 9, A at 11, and L at 61.

These comparisons also identified some positions within the TZF domains that can tolerate significant amino acid diversity. For example, P, T and R were present at position 9, E and S at position 11, N, S and T at 13, A, S, T, R, and F at 15, D, E, A, and N at 20, I, F, L, P and K at 28, H, G, I, and S at 29, etc. Less diversity is evident at other positions; for example, only hydrophobic residues were present at positions 10, 48 and 49, and only basic residues were present at position 21.

#### **Figure Legends for Example 4.**

##### **Fig. 2. Alignments of human CCCH zinc finger proteins.**

The three known human (h) CCCH proteins hTTP ((21)GenBank accession number M63625), hsERF1 (11B) ((14)accession number X71901), and hsERF2 (11D) ((16) accession number X78992) sequences were aligned using ClustalW Alignments (MacVector 6.5, Oxford Molecular) with an open gap penalty of 10.0 and an extended gap penalty of 0.05. The shaded areas indicate amino acid identity. The closely related double zinc finger domains are underlined, with the key cysteine and histidine residues indicated by dots under the sequence.

##### **Fig. 3A and 3B. UV cross-linking assays of TTP and related proteins with mutant mouse TNF-ARE probes.**

**Fig. 3A:** Cytosolic extracts of 293 cells transfected with either vector alone or constructs expressing the CCCH zinc finger proteins were prepared as described in Methods. Assays used <sup>32</sup>P-labeled m TNF ARE probes 1281-1350, 1309-1332, or a mutant of probe 1309-1332 (A/G), as indicated. Incubation of extracts (each sample in lanes 2, 3, 7, 8, 13 and 14 contained 5 µg of protein; lanes 4-6, 9-11, 15-17 contained 20 µg of protein) with 1.5 x 10<sup>6</sup> cpm of probe, UV cross-linking and RNase digestion were performed as described in Methods. Lanes 1, 12 and 18 (P', 1.5 x 10<sup>6</sup> cpm per sample): probe alone after digestion with RNase. Lanes 2, 6, 7, 11, 13 and 17 (BS+):

Extracts from 293 cells transfected with 5 µg of vector plasmid. Lanes 3-5: Extracts from 293 cells transfected with 1 µg /plate of plasmid CMV.hTTP.tag, CMV.CMG1.tag, or CMV.XC3H-3.tag, respectively; vector DNA was added to make the total transfected DNA 5 µg/plate. Extracts described in lanes 3-5 were also used for lanes 8-10 and lanes 14-16. The RNA-protein complexes were resolved by SDS-PAGE (12% gel) followed by autoradiography. The exposure time for the gel using probes 1281-1350 and 1309-1332 was 4 h at -70°C, and was 8h for the gel using 1309-1332 (A/G). The positions of molecular weight standards are indicated to the left of the gel.

**Fig. 3B:** The sequences of the probes used for the experiments described in Fig. 3A are shown; the adenosine residues mutated to guanosine residues in probe 1309-1332 (A/G) are indicated by the triangles.

**Fig. 4. Alignments of XC3H-4-like CCCH zinc finger proteins.**

The three known proteins that contain four CCCH zinc fingers were aligned using ClustalW Alignments (MacVector 6.5, Oxford Molecular) with an open gap penalty of 10.0 and an extended gap penalty of 0.05. The shaded areas indicate amino acid identity, the outlined areas indicate similarities. The closely related tandem zinc finger domains are underlined, with the key cysteine and histidine residues indicated by dots under the sequence. The sequences are: Carp CTH1 ((35) GenBank accession number CAA71245.2); Zebrafish CTH1 ((34) GenBank accession number CAB55775.1); Xenopus XC3H-4 ((20) GenBank accession number AAD24210).

**Fig. 5A and 5B: Alignment of tandem zinc finger domains of known CCCH proteins.**

The 64 amino acid TZF domains from the proteins described in the text were aligned with the Pileup function from GCG.

**Fig. 5A** shows a dendrogram is shown in which only the 64 amino acid TZF domains shown in 5B were used to calculate sequence similarities. The four major groupings are indicated.

**Fig. 5B** shows the alignment of the TZF domains. Identical amino acids are shaded in black; related amino acids are shaded in gray. The accession numbers for the proteins listed are as follows; for the cMG1 group: rat cMG1, X52590; human cMG1, X71901; mouse cMG1, P23950; and *Xenopus* XC3H-2, AAD24208. For the tis11d group: human TIS11d, X78992; mouse tis11d, P23949; *Xenopus* XC3H-3.1, AAD24209; and *Xenopus* XC3H-3.23. For the TTP group: human TTP, P26651; bovine TTP, P53781; mouse TTP, P22893; rat TTP, P47973; and *Xenopus* XC3H-1, AAD24207. For the XC3H-4 group: carp CTH1, CAA71245.2; zebrafish CTH1, CAA76889; and *Xenopus* XC3H-4, AAD 24210.

10

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### Incorporation by Reference

Throughout this application, various publications, patents, and/or patent applications are referenced in order to more fully describe the state of the art to which this invention pertains. The disclosures of these publications, patents, and/or patent applications are herein incorporated by reference in their entireties to the same extent as if each independent publication, patent, and/or patent application was specifically and individually indicated to be incorporated by reference.

### Other Embodiments

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

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